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Remarks

The Amendments

Independent claims 9 and 17 have been amended to recite "at least 13 contiguous nucleotides." New claims 63-82 recite single-stranded polynucleotides that comprise 14, 15, 20, 25, or 50 contiguous nucleotides. Paragraph [27] of the specification supports these recitations: "Single-stranded polynucleotides can contain any length from 12 contiguous nucleotides to a full-length sequence (*e.g.*, 12, 13, 14, 15, 20, 25, 50, 75, 100, 250, or 500 or more contiguous nucleotides)."

Each of independent claims 9, 17, 59, and 61 recites that the single-stranded polynucleotide is suitable for use as a probe to detect the SNP in or as a primer to amplify a portion of the gene that comprises the SNP. This recitation is supported in paragraph [28]: "Single-stranded polynucleotides of the invention can be used as probes or as primers."

Independent claim 17 has been amended to incorporate the recitations of dependent claim 19.

New claims 59-62 are supported by paragraph [33] and by originally filed claims 19, 20, 37, and 39.

The amendments to the claims add no new matter.

Examination of Other Species

The pending claims have been examined with respect to the SNP at position 243 of SEQ ID NO:1. If these claims are found allowable with respect to the elected species of SNP, Applicant respectfully requests that the patentability of the other recited SNPs be considered. M.P.E.P. § 809.02(e).

The Rejection of Claims 9, 10, 14, and 15 Under 35 U.S.C. § 102(b)

Claims 9, 10, 14, and 15 stand rejected under 35 U.S.C. § 102(b) as anticipated by Halverson *et al.*, U.S. Patent 5,874,217 ("Halverson"). Applicants respectfully traverse the rejection.

To reject a claim as anticipated, each and every element as set forth in the claim must be either expressly or inherently described in a single prior art reference. *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 U.S.P.Q.2d (BNA) 1051, 1053 (Fed. Cir. 1987). Halverson is cited as teaching a primer (SEQ ID NO:22) of which nucleotides 1-12 are identical to nucleotides 236-247 of SEQ ID NO:1 if SEQ ID NO:1 were to comprise a SNP at position 243 (*i.e.*, C instead of T). Office Action at page 3, second to last paragraph. Independent claim 9 has been amended to recite "at least 13 contiguous nucleotides." Halverson's SEQ ID NO:22 does not comprise the recited at least 13 contiguous nucleotides. Thus, Halverson does not anticipate amended claim 9 or dependent claims 10, 14, and 15.

Applicants respectfully request withdrawal of the rejection.

The Rejection of Claim 17 Under 35 U.S.C. § 102(b)

Claim 17 stands rejected under 35 U.S.C. § 102(b) as anticipated by pages 12 and 28 of the 1996/1997 New England Biolabs Catalog ("NEB"). Applicants respectfully traverse the rejection.

Claim 17 is directed to a kit. The kit comprises a single-stranded polynucleotide comprising at least 13 contiguous nucleotides of a human surfactant protein C gene, wherein the at least 13 contiguous nucleotides comprise a SNP, wherein the SNP is associated with an interstitial lung disease and wherein the single-stranded polynucleotide is suitable for use as a

probe to detect the SNP or as a primer to amplify a portion of the gene that comprises the SNP. The kit also comprises instructions for a method of screening an individual for a predisposition to developing the interstitial lung disease. The method comprises the step of assaying a biological sample obtained from the individual to determine if an allele of the individual's surfactant protein C gene comprises the SNP.

The Office Action cites NEB as teaching a kit comprising the restriction endonuclease BstNI and directions for its use. Office Action at page 4, fourth paragraph. The Office Action asserts that BstNI inherently is a reagent for detecting the SNP present at the intronic nucleotide immediately 3′ of nucleotide 460.

To reject claim 17 as anticipated by NEB, NEB must either expressly or inherently describe each and every element set forth in claim 17. *Verdegaal Bros.*, 814 F.2d at 631, 2 U.S.P.Q.2d (BNA) at 1053. Claim 17 has been amended to delete the recitation "a reagent for detecting a SNP in a mutant allele." The kit of claim 17 as amended comprises the single-stranded polynucleotide and the recited instructions. NEB discloses neither of these elements. Thus, NEB does not anticipate the subject matter of amended claim 17.

For the same reason, the rejection also does not apply to new claims 59-62. New claims 59-62 recite the same single-stranded polynucleotide recited in amended claim 17. New claims 63-82 recite single-stranded polynucleotides comprising at least 14, 15, 20, 25, or 50 contiguous nucleotides. NEB does not disclose these polynucleotides.

Applicants respectfully request withdrawal of the rejection.

The Rejection of Claim 16 Under 35 U.S.C. § 103(a)

Claim 16 stands rejected under 35 U.S.C. § 103(a) as obvious over Halverson in view of Adams *et al.*, U.S. Patent 5,641,658 ("Adams"). Applicants respectfully traverse the rejection.

The U.S. Patent and Trademark Office bears the initial burden of establishing a *prima facie* case of obviousness. The *prima facie* case requires three showings:

> First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations.

Manual of Patent Examining Procedure, 8th ed. (M.P.E.P.), § 2142. In the present application, a *prima facie* case that claim 16 is obvious has not been made because the cited combination of references does not disclose each element in claim 16.

Dependent claim 16 is directed to "the single-stranded polynucleotide of claim 9 which is bound to a solid support." Claim 9 has been amended to recite "at least 13 contiguous nucleotides of a human surfactant protein C gene, wherein the at least 13 contiguous nucleotides comprise a SNP associated with an interstitial lung disease that is associated with a defect in surfactant protein C."

Halverson is cited as teaching a single-stranded polynucleotide comprising 12 contiguous nucleotides of the recited human surfactant protein C gene. Page 5, last full paragraph. Adams is cited as teaching primers bound to solid supports. Page 6, last paragraph. Neither Halverson nor Adams discloses the single-stranded polynucleotide recited in amended independent claim 9. Thus, the combination of Halverson and Adams does not disclose each element recited in dependent claim 16.

The Office Action has not made *prima facie* case of that claim 16 as amended is obvious. Applicants respectfully request withdrawal of the rejection.

The Rejection of Claims 17, 19, and 20 Under 35 U.S.C. § 103(a)

Claims 17, 19, and 20 stand rejected under 35 U.S.C. § 103(a) as obvious over Halverson in view of Ahern, *The Scientist 9*, page 20, July 1995 ("Ahern"). Claim 19 has been canceled. Applicants respectfully traverse the rejection of claims 17 and 20.

As with the rejection of claim 16 discussed above, the obviousness rejection of claims 17 and 20 is based on the assertion that Halverson teaches the recited single-stranded polynucleotide. Page 7, second full paragraph. Independent claim 17 has been amended to recite "a single-stranded polynucleotide comprising at least 13 contiguous nucleotides." Halverson does not disclose such a single-stranded polynucleotide. Nor does Ahern contain any teaching of the single-stranded polynucleotide recited in amended claim 17. In addition, neither reference contains any teaching or suggestion of the method described in the recited instructions.

The Office Action has not made a *prima facie* case that claims 17 and 20 are obvious because the combination of Halverson and Ahern does not teach each element recited in claims 17 and 20.

Applicants respectfully request withdrawal of the rejection.

The Rejection of Claims 9, 10, 14-17, 19, and 20 Under 35 U.S.C. § 112, first paragraph

Claims 9, 10, 14-17, 19, and 20 stand rejected under 35 U.S.C. § 112, first paragraph, as not enabled. Claim 19 has been canceled. Applicants respectfully traverse the rejection of claims 9, 10, 14-17, and 20.

To satisfy 35 U.S.C. § 112, first paragraph, "the scope of the claims must bear a *reasonable correlation* to the scope of enablement provided by the specification to persons of ordinary skill in the art." *In re Fisher*, 427 F.2d 833, 839, 166 U.S.P.Q. (BNA) 18, 24 (C.C.P.A. 1970) (emphasis added). Any experimentation needed to practice the invention must not be undue. *In re Wands*, 858 F.2d 731, 736-37, 8 U.S.P.Q.2d (BNA) 1400, 1404 (Fed. Cir. 1988) ("The key word is 'undue,' not 'experimentation.'"). The teachings of the present specification meet these standards.

Claims 9, 10, 14-17, and 20, and new claims 59-82 recite a SNP in a human surfactant protein C gene that is "associated with an interstitial lung disease." The specification as a whole teaches the association of particular SNPs with interstitial lung disease. *See, e.g.*, the title ("Single Nucleotide Polymorphisms Associated with Interstitial Lung Disease"); paragraph [22]: "Detection of these SNPs can be used to identify individuals who have a predisposition for developing an interstitial lung disease . . . ."; see also Tables 1 and 2, which teach 17 SNPs that are found only in patients with lung disease. Each of the 17 disease-associated SNPs disclosed in the specification causes a detectable defect in the surfactant C protein. See column 5 of Tables 1 and 2. The specification teaches several mechanisms by which surfactant protein C's involvement in interstitial lung disease may occur. *See* paragraphs [04] – [08].

The specification also enables those skilled in the art to identify additional SNPs falling within the scope of claims 9, 14-17, 59, and 61 and new claims 63-82 without undue

experimentation. The specification teaches that "[a]ssociation of a SNP with interstitial lung disease can be determined, for example, by statistical correlation of a disease phenotype with a particular SNP. Such methods are well known in the art and are routinely carried out by practitioners in the art. *See, e.g.*, Curtis *et al.*, Ann Hum Genet 2001 Jan;65(Pt 1):95-107 Johansson *et al.*, Genes Immun 2000 Aug;1(6):380-5; Johnson *et al.*, Genes Immun 2001 Aug;2(5):273-5." *See also* Hacker, *et al.*, *Gut 40*, 623-27, 1997 ("Hacker") cited in the Office Action.

One skilled in the art would not need to undertake experimentation at all to use the polymorphisms recited in claims 10, 20, 60, and 62: the specification already teaches that the recited SNPs are associated with interstitial lung disease. See, *e.g.*, Tables 1 and 2 and paragraph [22]. The need for routine screening for SNPs in the surfactant protein C gene and their correlation with interstitial lung disease does not mean claims 9, 14-17, 59, and 61 and new claims 63-82 are not enabled.

In fact, post-priority date[1] publications have confirmed the association of defects in surfactant protein C with interstitial lung disease and have identified additional disease-associated mutations in the surfactant protein C gene. These publications demonstrate that it is not at all unpredictable to identify such mutations.

Amin *et al.*, "Surfactant protein deficiency in familial interstitial lung disease," *J. Pediatrics 139*, 85-92, July 2001 ("Amin"; attachment 1) demonstrated that the absence of surfactant protein C is associated with familial interstitial lung disease. Page 85, abstract. Amin thus supports the association of surfactant protein C and interstitial lung disease.

[1] The application claims and is entitled to a priority date of February 14, 2001.

Thomas *et al.*, "Heterozygosity for a Surfactant Protein C Gene Mutation Associated with Usual Interstitial Pneumonitis and Cellular Nonspecific Interstitial Pneumonitis in One Kindred," *Am. J. Respir. Crit. Care Med. 165*, 1322-28, 2002 ("Thomas"; attachment 2), confirms that "[t]here is increasing evidence that implicates SP-C in the pathophysiology of some types of chronic lung disease." Page 1326, col. 1, lines 14-15. Thomas identified an additional SP-C gene mutation not disclosed in the present specification: a "heterozygous exon 5 + 128 T→A transversion" in a large kindred exhibiting two different pathological diagnoses. Page 1322, abstract. The transversion substitutes glutamine for leucine at position 188 of the C-terminal region of pro-SP-C. Page 1324, paragraph bridging columns 1 and 2. Thomas teaches that "[t]he presence of two different pathological diagnoses in affected relatives sharing this mutation indicates that in this kindred, these diseases may represent pleiotropic manifestations of the same central pathogenesis." Page 1322, abstract.

Nogee *et al.*, "Mutations in the Surfactant Protein C Gene Associated With Interstitial Lung Disease," *Chest 121*, 20S-21S, 2002 ("Nogee"; attachment 3), discusses the two mutations that occur in the intronic nucleotide immediately 3′ of nucleotide 460 (460 +1) (see Table 1 of the present application). The G→T substitution was identified as a *de novo* mutation, whereas the G→A mutation was present in infants with familial interstitial lung disease. Page 20S. Both mutations cause a deletion of exon 4. *Id.* Nogee states that "[t]he occurrence of a *de novo* mutation functionally identical to a familial mutation in infants with the same phenotype strongly supports the hypothesis that the mutations were causally related to their lung disease." Sentence bridging pages 20S and 21S.

Pantelidis *et al.*, "Surfactant gene polymorphisms and interstitial lung diseases," *Resp. Res. 3*, 14-20, 2002 ("Pantelidis"; attachment 4), confirms that identifying polymorphisms in

surfactant protein genes, including the gene for surfactant protein C, is an accepted and workable approach for understanding the genetics of interstitial lung disease. Page 16, col. 1, line 13, to col. 2, second paragraph from bottom. Pantelidis discusses the G→A mutation that causes the deletion of exon 4, as well as the existence of other SPC mutations personally reported to the authors by Dr. Nogee. Page 16, last paragraph, to page 4, last paragraph of col. 1.

Hamvas *et al.*, "Progressive lung disease and surfactant dysfunction with a deletion of surfactant protein C gene," *Am. J. Respir. Cell Mol. Biol.*, e-pub ahead of print, December 4, 2003 ("Hamvas"; attachment 5), states definitively that "[m]utations in the surfactant protein-C (SP-C) gene are responsible for familial and sporadic interstitial lung disease (ILD)." Page 2, abstract. Hamvas identified an additional disease-associated mutation: "an in-frame 9-base pair deletion spanning codons 91-93 in Exon 3 of the Sp-C gene." *Id.* While this mutation is not a point mutation (SNP), it confirms the association of surfactant protein C mutations generically with interstitial lung disease.

Taken together, these post-priority date publications confirm the teachings of the specification that surfactant protein C mutations are associated with interstitial lung disease, that identification of such mutations can be readily and routinely performed using well-known techniques, and that identification of additional mutations is not unpredictable.

The Office Action asserts generally that associating SNPs with a disease state is unpredictable and cites four publications to support this assertion: Hacker, Pennisi, *Science 281*, 1788-89, 1998 ("Pennisi"), Blumenfeld *et al.*, WO 99/52942 ("Blumenfeld"), and the post-priority date publication Hirschhorn *et al.*, *Genetics in Medicine 4*, 45-61, March/April 2002 ("Hirschhorn"). Viewed in light of the specification's teachings and the confirming teachings of

the art discussed above, none of these publications actually supports a *prima facie* case of non-enablement.

Hacker is cited as disclosing a study that was unable to confirm an association between ulcerative colitis and a SNP previously identified to be associated within a different population. Sentence Bridging pages 13 and 14 of the Office Action. In contrast, the present specification teaches that the disclosed surfactant C protein SNPs "can be used to identify individuals who have a predisposition for developing an interstitial lung disease . . . ." Paragraph [22]. Post-priority date publications support this teaching. The Office Action provides no reason to think that the association between the disclosed SNPs and interstitial lung disease occurs only in specific populations. Hacker is particularly irrelevant to claims 10, 20, 60, and 62, each of which recites particular SNPS that the inventors already have determined are disease-associated. See Tables 1 and 2.

Pennisi is cited as teaching that it is difficult to associate SNPs with disease states or to identify key genes as associated with a disease. Office Action at page 14, lines 3-8. This teaching is not relevant to claims 10, 20, 60, and 62, each of which recites particular disease-associated SNPs.

Pennisi also is not relevant to generic claims 9, 14-16, 59, or 61 or to new claims 63-82. None of the claims is directed to identifying key genes as associated with a disease; the surfactant protein C gene is taught in the present application to be involved in interstitial lung disease. As discussed above, those skilled in the art have not had difficulty associating mutations in the surfactant protein C gene with interstitial lung disease. *See, e.g.*, Thomas and Hamvas.

Blumenfeld is cited as teaching that some polymorphisms in the FLAP gene are associated with asthma whereas others are not. Office Action at page 14, lines 9-12. Blumenfeld's teaching that some polymorphisms in the FLAP gene are not associated with asthma is not relevant because the pending claims recite only SNPs that are associated with an interstitial lung disease. Blumenfeld's teaching certainly is not relevant to claims 10, 20, 60, and 62, which recite particular SNPs that the specification teaches are associated with lung disease.

Hirschhorn, a post-priority date publication, is cited as "reiterat[ing] the unpredictability of determining an association between a SNP and a disease state." Office Action at page 14, last paragraph. The Office Action has, however, provided no reason to doubt that SNPs in the human surfactant protein C gene can be associated with an interstitial lung disease, as taught in the specification and as confirmed in the post-priority date publications discussed above. In fact, the associations of 17 particular SNPs with lung disease that are reported in Tables 1 and 2 of the specification, as well as the additional SP-C gene mutations taught in Thomas and Hamvas, refute the Office Action's assertion of unpredictability. Hirschhorn does not specifically address surfactant protein C, and the record evidence refutes Hirschhorn's general statements.

None of the cited references supports a conclusion that claims 9, 10, 14-17, 20, and new claims 59-82 are not enabled. In characterizing the art as "highly unpredictable," the Office Action has not properly weighed the teachings of the specification and the relative skill of those practicing in this art. As evidenced by the articles discussed above, those skilled in the art are able and prepared to carry out the statistical correlations needed to correlate a particular SNP in the surfactant protein C gene with an interstitial lung disease. Carrying out such methods is routine to practitioners in this art and would therefore not be considered undue experimentation.

Publications such as Thomas and Hamvas, discussed above, demonstrate that identification of additional disease-associated mutations is not unpredictable.

To determine enablement, the examiner must weigh all the evidence, including the teachings of the specification. M.P.E.P. § 2164.05(a). In this case, the U.S. Patent and Trademark Office has not given sufficient weight to the teachings of the specification or the level of skill of practitioners in the art, as evidenced by the post-priority date publications discussed above. Considering the evidence discussed above, the rejection should be withdrawn. These arguments apply with equal force to new claims 59-82.

Applicants respectfully request withdrawal of the rejection.

The Rejection of Claims 9, 10, 14-17, 19, and 20 Under 35 U.S.C. § 112, first paragraph

Claims 9, 10, 14-17, 19, and 20 stand rejected under 35 U.S.C. § 112, first paragraph, as not sufficiently described in the specification. Claim 19 has been canceled. Applicants respectfully traverse the rejection of claims 9, 10, 14-17, and 20.

The first paragraph of 35 U.S.C. § 112 requires that the specification provide a written description of the claimed invention:

> [t]he specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

The purpose of the written description requirement is to ensure that the specification conveys to those skilled in the art that the applicants possessed the claimed subject matter as of the filing date sought. *Vas-Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1563-64, 19 U.S.P.Q.2d (BNA) 1111,

1117 (Fed. Cir. 1991). The specification must be considered as a whole when determining whether the written description requirement is met. *In re Wright*, 866 F.2d 422, 425, 9 U.S.P.Q.2d (BNA) 1649, 1651 (Fed. Cir. 1989). As a whole, the specification describes the subject matter of amended claims 9, 10, 14-17, and 20. The specification in its entirety also describes the subject matter of new claims 59-82.

There is no basis for a written description rejection of claims 10, 20, 60, and 62, each of which recites particular SNPs that are taught in the specification to be disease-associated. No evidence or allegation has been presented that these SNPs are not disease-associated.

Each of claims 9, 14-16, 59, 61, and 63-82 recites a genus of single-stranded polynucleotides "comprising at least 13 contiguous nucleotides of a human surfactant protein C gene, wherein the at least 13 contiguous nucleotides comprise a SNP associated with an interstitial lung disease." Written description of a genus of nucleic acid molecules may be achieved by sufficiently describing a representative number of species within the genus. *University of California v. Eli Lilly and Co.*, 119 F.3d 1559, 1569, 43 U.S.P.Q.2d (BNA) 1398, 1406 (Fed. Cir. 1997). The specification provides a wild-type coding sequence for the human surfactant protein C gene and describes 17 specific SNPs within the recited genera. The Office Action cites no basis for concluding that this disclosure does not provide a representative number of species within the recited genera. Even in an "unpredictable art" applicants "are *not* required to disclose *every* species encompassed by their claims . . . ." *In re Angstadt*, 537 F.2d 498, 502-03, 190 U.S.P.Q. (BNA) 214, 218, (C.C.P.A. 1976) (emphasis in original). Otherwise, a specification would have to describe explicitly every species within a claimed genus; that is not the law. *See Engel Indus., Inc. v. Lockformer Co.*, 946 F.2d 1528, 1531, 20 U.S.P.Q.2d (BNA) 1300, 1302 (Fed. Cir. 1991).

The Office Action faults the claims for not requiring that the recited contiguous nucleotides overlap with the "mutant SNP." Office Action at paragraph bridging pages 17 and 18. Each of the pending claims explicitly recites that the contiguous nucleotides comprise the SNP that is associated with the interstitial lung disease. The SNP itself is the relevant, disease-associated mutation; see paragraph [26]: "[a] 'mutant allele' of a human SP-C gene comprises at least one SNP with respect to SEQ ID NO:1, which is associated with interstitial lung disease." The claims do not require that the human surfactant protein C gene contain any mutation other than the recited SNP. To clarify this point, the recitation "of a mutant allele" has been deleted.

The Office Action faults claim 17 for including "reagents that detect any polymorphism within the human surfactant C gene that is associated with interstitial lung disease." Page 20, first full paragraph. To advance prosecution, claim 17 has been amended to delete this recitation.

The Office Action also asserts that the claims encompass SNPs that are associated with any interstitial lung diseases, which have in common that they are diseases of the lung interstitia but otherwise represent a diverse group of diseases that have different causes and etiologies. Office Action at page 18, lines 9-15. The etiology of the recited interstitial lung disease is not relevant to the claimed subject matter. The claims merely require that the recited SNP in the human surfactant protein C gene be associated with an interstitial lung disease. In fact, the post-priority date publications discussed above in connection with enablement discuss "interstitial lung disease" generally and indicate that the same SP-C gene mutation can be present in different types of interstitial lung diseases. *See, e.g.*, Thomas at page 1322, abstract: "[t]he presence of two different pathological diagnoses in affected relatives sharing this mutation indicates that in this kindred, these diseases may represent pleiotropic manifestations of the same central pathogenesis."

The Office Action asserts that the recitation of a single-stranded polynucleotide "comprising" the recited contiguous nucleotides renders the claims too broad for the specification to support. To advance prosecution, each of the independent claims now recites that the single-stranded polynucleotide is suitable for use as a probe to detect the SNP or as a primer to amplify a portion of the gene that comprises the SNP. Thus, the claimed single-stranded polynucleotides encompass the recited contiguous nucleotides of the human surfactant protein C gene and any other nucleotides that do not interfere with the use of the claimed polynucleotides as probes or primers.

Finally, the Office Action asserts that "[t]he 'functional language' that defines these SNPs as being associated with interstitial lung disease does not lead one to a predictable structure that would allow one to determine even which of the 32 polymorphisms disclosed in the specification meet the requirement of being 'associated with interstitial lung disease,' let alone any SNP that has not been described explicitly in the specification." Page 20, lines 15-19. First, the specification explicitly teaches which of the SNPs disclosed in Tables 1 and 2 are associated with lung disease; these are the SNPs identified with a "+" sign in the first column of the tables. Second, whether a SNP has a "predictable structure" is not relevant to written description of the claimed subject matter. The specification describes the coding sequence of the human surfactant protein C gene and teaches 17 mutations in the gene that are correlated with interstitial lung disease. The application thus discloses a sufficient number of representative species to support the subject matter of claims 9, 14-16, 59, 61, and 63-82, which do not recite particular SNPs.

The specification fully supports claims 9, 10, 14-17, and 20 as amended as well as new claims 59-82. Applicants respectfully request withdrawal of this rejection.

Respectfully submitted,

Date: December 23, 2003

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Attachment 1

Surfactant protein deficiency in familial interstitial lung disease

Raouf S. Amin, MD, Susan E. Wert, PhD, Robert P. Baughman, MD, Joseph F. Tomashefski, Jr, MD, Lawrence M. Nogee, MD, Alan S. Brody, MD, William M. Hull, MS, and Jeffrey A. Whitsett, MD

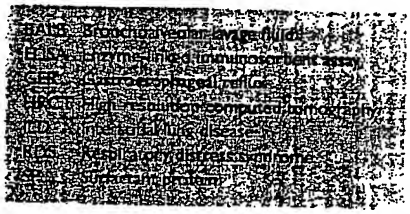
Objective: To determine the contribution of surfactant protein abnormalities to the development of chronic lung injury in a familial form of interstitial lung disease.

Study design: An 11-year-old girl, her sister, and their mother who were diagnosed with chronic interstitial lung disease underwent laboratory investigation of surfactant protein expression in bronchoalveolar lavage fluid and lung biopsy specimens. Nineteen patients with idiopathic pulmonary fibrosis and 9 patients who were investigated for pulmonary malignancy but who did not have interstitial lung disease served as control subjects.

Results: The 3 family members were found to have absent surfactant protein C (SP-C) and decreased levels of SP-A and SP-B in bronchoalveolar lavage fluid (BALF). Immunostaining for pulmonary surfactant proteins in lung biopsy specimens obtained from both children demonstrated a marked decrease of pro-SP-C in the alveolar epithelial cells but strong staining for pro-SP-B, SP-B, SP-A, and SP-D. No deviations from published surfactant protein B or C coding sequences were identified by DNA sequence analysis. All control subjects had a detectable level of SP-C in the BALF.

Conclusion: The apparent absence of SP-C and a decrease in the levels of SP-A and SP-B are associated with familial interstitial lung disease. (J Pediatr 2001;139:85-92)

Chronic interstitial lung disease in children comprises a large group of pulmonary disorders characterized by variable degrees of infiltration of distal airspaces with inflammatory cells. The diseases cause considerable degrees of morbidity and mortality. ILD in children presents with a wide spectrum of histologic abnormalities that usually do not fit the classification for ILD used in the adult population. A hereditary form of ILD has been suggested by reports of family clustering within and across generations.[1-3] The inflammatory process in ILD begins with an initial injury to the alveolar and interstitial structures (alveolitis), which is followed by a stage of tissue repair and variable degrees of fibrosis. The inciting cause and the mechanisms of propagation of alveolitis and of its progression to fibrosis are not completely understood. In



ILD, however, alveolar proteinosis may precede the development of pulmonary fibrosis.[4] The accumulation of proteinaceous material in the alveolar space is a characteristic finding in lung diseases associated with surfactant system abnormalities.[5-7] Pulmonary surfactant is a complex surface-active mixture of phospholipids and proteins found in the fluid lining the alveolar surface. Surfactant contains unique proteins designated surfactant protein A, SP-B, SP-C, and SP-D. Selective deficiency of SPs disturbs the biophysical function of surfactant and leads to alveolar accumulation of proteins and lipids. We therefore hypothesized that in a hereditary form of pulmonary fibrosis with a mild degree of alveolar proteinosis, a deficiency of SP could be shared among the affected family members and could have contributed

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to the development of the disease. A mother and her 2 daughters with ILD were found to have a marked decrease in the level of pro-SP-C in alveolar epithelial cells and an undetectable level of SP-C in the bronchoalveolar lavage fluid. A selective absence of SP-C in familial pulmonary fibrosis indicates that SP-C deficiency may be associated with chronic ILD.

METHODS

Case Histories

Patient I, an 11-year-old girl, was given a diagnosis of ILD at the age of 7 years. Her family reported that for several years she seemed to tire easily with exertion. There was no history of cough, chest pain, fever, or recurrent sinopulmonary infections. There was also no evidence of cardiac, dermatologic, gastrointestinal, hepatic, immunologic, renal, or skeletal disorders. The patient was born 5 weeks prematurely, weighing 2250 g, to a 20-year-old mother. She remained in the hospital for 3 weeks because of an oxygen requirement and poor weight gain. She had pneumonia at the age of 18 months for which she was hospitalized for 2 weeks. Despite a normal appetite, she remained small for her age. At the time of presentation, she was not receiving any medication. She was residing in an urban area in the state of Indiana, away from her mother and sister who were residing in Florida. There was no history of any environmental exposure. ILD was diagnosed by means of radiographs and lung biopsy. At the time of diagnosis, the patient required oxygen, 2½ L/min, to maintain normal oxygen saturation. She was treated initially with systemic steroids and experienced only short-term improvement. Treatment with hydroxychloroquine led to complete weaning from oxygen. There was no history of chronic lung disease in the maternal grandparents, but history for the paternal grandparents was not available.

Patient II is the mother of patients I and III. She was given a diagnosis of ILD when she was 26 years old and 4 months' pregnant with patient III. For the previous 6 years, she had intermittent episodes of shortness of breath. One year before the initial evaluation, she had a laparoscopic examination and developed shortness of breath during the postoperative recovery period. She subsequently developed persistent pulmonary infiltrates, which were thought to be due to fibrotic changes. During her second pregnancy, she became increasingly dyspneic, and 2 days after delivery of patient III, she developed acute respiratory failure for which she required mechanical ventilation. She was treated aggressively with antibiotics and corticosteroids and was weaned from the ventilator. A diagnosis of idiopathic pulmonary fibrosis was made by computed tomography scan. The patient refused to undergo a lung biopsy. Her condition improved during treatment with high-dose corticosteroids over the next year, and she was weaned from oxygen. She currently receives a maintenance dose of corticosteroids and has stable pulmonary function. For the past 2 years, her pulmonary function tests showed a forced vital capacity of 1.9 L (60% predicted), forced expiratory volume in 1 second of 1.42 L/s (56%) predicted, and carbon monoxide diffusion of 6 mL/min/mm Hg (35% predicted).

Patient III is the 4-year-old daughter of patient II and half sister of patient I. She was born at 35 weeks' gestation weighing 2280 g. At birth, she developed respiratory distress syndrome and required mechanical ventilation for a period of 2 weeks. At 3½ weeks of age, she was given a diagnosis of lymphocytic interstitial pneumonitis secondary to *Pneumocystis carinii*, based on findings from an open lung biopsy. She was intubated for 2 weeks and was discharged home receiving oxygen, 0.125 L/min. She developed enteroviral pneumonitis at 10 months of age, respiratory syncytial virus bronchiolitis at 15 months of age, and parainfluenza type III bronchiolitis at 18 months of age. At 4 months of age, she was found to have gastroesophageal reflux and was treated surgically with fundoplication and placement of a gastrostomy tube. A second fundoplication was performed at 13 months of age. There was no evidence of an immune deficiency disorder, including a negative human immune deficiency virus test result on repeated occasions. Repeated radiographic studies demonstrated a slowly progressive ILD. She showed only a short-term response to systemic steroids and is currently treated with hydroxychloroquine.

Control Subjects

GROUP A. BALF samples were obtained from 5 subjects (3 men and 2 women; mean age, 57 years; range, 43-69 years) who underwent flexible bronchoscopy and bronchoalveolar lavage as a part of an evaluation for localized pulmonary malignancy, and these served as control samples in an enzyme-linked immunosorbent assay of SPs A, B, C, and D. Four BALF samples were used for Western blot analysis of all SPs. The lavage was performed in an unaffected lobe.

GROUP B. BALF samples were obtained from 10 patients (6 men and 4 women; mean age, 61 years; range, 47-72 years) who were given a diagnosis of idiopathic pulmonary fibrosis and underwent flexible bronchoscopy as a part of the evaluation for ILD, and these served as control samples in an ELISA of SPs A, B, C, and D. BALF samples were also used for Western blot analysis of SP-C.

GROUP C. Lung biopsy specimens from 13 adult patients were immunostained for SP-A, pro-SP-B, SP-B, pro-SP-C, and SP-D. Nine patients had idiopathic pulmonary fibrosis, and 4 patients had lung biopsy as part of an evaluation for localized pulmonary malignancy.

Radiographic Studies

High-resolution computed tomography was performed with the following

technique: 1-mm sections were scanned at 10-mm intervals from the lung apices to the lung bases at full inspiration, and 1-mm sections were scanned at 20-mm intervals in expiration.

Bronchoalveolar Lavage

Flexible bronchoscopy was performed with general anesthesia in the 2 pediatric patients and with conscious sedation in the adult subjects. The bronchoscope was advanced to a wedge position in one of the pulmonary segments. The isolated segment was lavaged with 2 aliquots that consisted of 1 mL/kg normal saline solution for pediatric patients and 60 cc for the adult subjects. Samples were dialyzed against 3 mmol/L Tris pH 7.4 with the use of Spectrapore membrane (Spectrum Laboratories, Inc, Rancho Domingues, Calif) in the molecular weight range of 6000 to 8000 d. SP-C forms a large aggregate and is not affected by dialysis. Samples were frozen at –30°C until analyzed.

Histopathology

Lung biopsy specimens were obtained from patient I at the time of the initial evaluation and from patient III at the time of infection with *P carinii*. No lung biopsy specimen was obtained from patient II. Paraffin sections were stained with hematoxylin and eosin for histopathologic analysis. Trichrome and elastin stains were used for identification and localization of collagen and elastic fibers. A periodic acid-Schiff stain, with and without diastase digestion, was performed for the detection of alveolar proteinosis material, and an iron stain was performed for hemosiderin deposits. Gomori methenamine silver staining was performed for the detection of *P carinii*. Additional histochemical and immunohistochemical stains were performed for mycobacteria, adenovirus, Epstein-Barr virus, herpes simplex virus types I and II, parvovirus, and cytomegalovirus. Cytospins of BALF from patients I, II, and III were stained with hematoxylin and eosin, Gomori methenamine silver, Giemsa stain, and oil red O and with Perl solution for iron staining.

Immunohistochemistry

ANTISERA. A panel of rabbit polyclonal antibodies specific for SP-A, SP-B, pro-SP-B, pro-SP-C, and SP-D was used to screen these samples for the expression of surfactant-associated proteins, as previously described.[8-11] Rabbit polyclonal antibody to human recombinant SP-C, whose amino acid sequence was altered by replacing the cysteines in positions 4 and 5 with phenylalanine and methionine and in position 32 with isoleucine, was used.[12]

IMMUNOHISTOCHEMISTRY. Formalin-fixed, paraffin-embedded biopsy samples were received for evaluation. Five-micron–thick sections were cut on a rotary microtome and loaded onto polysine-coated slides (Fisher, Atlanta, Ga). A Vectastain ABC Peroxidase Elite Rabbit IgG kit (Vector Laboratories, Inc, Burlingame, Calif) was used to detect the antigen/antibody complexes, as previously described.[9,10] Controls included (1) formalin-fixed, paraffin-embedded, surgical samples of previously stained, immunopositive, human neonatal or pediatric lung, which were re-stained at the indicated dilutions along with the test cases and (2) omission of the primary antibody to check for endogenous biotin and peroxidase activity, as well as nonspecific binding of the secondary antibody.

Immunoblot

Dialyzed BALF from the 3 patients and 4 control subjects from group A were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 10% to 20% gradient gel, transferred to nitrocellulose, and blotted with rabbit anti-SP-A, anti-SP-B, anti-SP-C,[13] and SP-D antibodies. BALF from 10 control subjects from group B were blotted with anti-SP-C only. Proteins were detected by using enhanced chemiluminescence and a horseradish peroxidase–conjugated second antibody (Amersham Life Sciences, Arlington Heights, Ill), followed by exposure to Kodak x-ray film for 30 seconds to 5 minutes. Results shown are representative of 2 independent experiments.

Sensitivity of SP-C immunoassay was determined by performing Western blot analysis with 8 concentrations of recombinant SP-C ranging from 12.5 ng to 0.0625 ng. Recombinant SP-C solution in a concentration of 2.5 μg/mL was serially diluted to the desired amounts in Laemmli sample buffer containing 50 μL/mL of β-mercaptoethanol.

Surfactants

SP-A was purified from the BALF of patients with alveolar proteinosis.[14] SP-B was isolated from alveolar lavage of adult sheep lungs through a 3-step centrifugation procedure that included a 0.8 mol/L sucrose gradient.[15] Recombinant SP-C was provided by ByK Gulden (Konstanz, Germany). A recombinant 34–amino acid human SP-C sequence, altered by replacement of cysteine in positions 4 and 5 with phenylalanine and methionine and in position 32 by isoleucine, was expressed in bacteria and purified.[12]

ELISA

Wells were coated overnight at 4°C with 100 μL of 0.1 mol/L sodium bicarbonate. After the plates were washed with a wash buffer, 100 μL of 82B buffer with 5% human albumin was added. The buffer was removed after 15 minutes, and 100 μL of standard at concentrations of 5, 10, 25, 50, 75, and 100 ng/mL of SP-B or μg/mL of SP-A and samples diluted in phosphate-buffered saline with NP-40 (Sigma Chemical Co, St Louis, Mo) were added. Plates were then incubated for 1 to 2 hours at 37°C. The contents of each well were washed 3 times with wash buffer, and 100 μL of polyclonal antibody diluted 1:1000 in 82b solution with 5% human albumin was added. The mixture was incubated at 37°C for 1 hour. After the contents were discarded, 100 μL of goat anti-

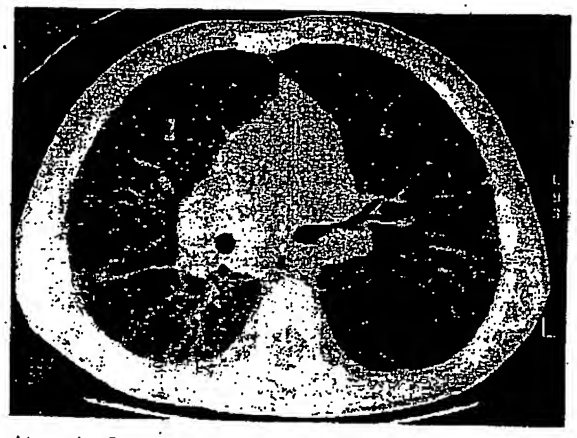

Fig 1. Radiographic study of patient III. HRCT of patient III shows both large and small areas of low attenuation with irregular thickening of intralobular septa. Coarse linear densities are seen in right lower lobe. Much of remaining lung shows diffuse ground-glass opacity.

rabbit IgG horseradish peroxidase conjugate, diluted in 1:1000 buffer with 5% albumin, was incubated for 1 hour at 37°C. The contents of each well were discarded and washed, and then 100 μL of substrate solution was added to each well. The absorbance was read at 492 nm after 100 μL of 50% sulfuric acid was added.

DNA Analysis

Genomic DNA was prepared from blood leukocytes with a commercially available kit (Puregene, Gentra Systems, Minneapolis, Minn) and analyzed for both known and possible novel *SP-B* gene mutations by use of heteroduplex analysis as previously described.[11] The *SP-C* gene was analyzed for possible mutations by direct sequencing of polymerase chain reaction products amplified from genomic DNA prepared from each affected child. Direct sequencing of polymerase chain reaction products included both alleles. The patient's SP-C genomic sequences were compared with published SP-C DNA sequences.[16,17]

RESULTS

Radiologic Studies

Chest radiographs demonstrated hyperinflation in all 3 patients. HRCT showed extensive areas of decreased attenuation. Expiratory images in patients I and III showed air trapping in these areas. This appearance reflects destructive changes in the parenchyma. The presence of hyperinflation and air trapping suggests that the predominant abnormality is emphysema or cystic lung disease. Ground-glass opacity was a predominant feature in patient III. Ground-glass opacity is a nonspecific finding but frequently suggests an active or evolving disease process (Fig 1).

Histopathology

PATIENT I. In patient I, there is variable interstitial fibrosis and moderately severe interstitial inflammation throughout the lung, consisting of lymphocytes, plasma cells, and peribronchial lymphoid aggregates. Lung architecture is distorted by lobular areas of dilated fibrous-walled airspaces. Fibrotic alveolar walls are diffusely lined by hyperplastic alveolar type II pneumocytes. The interstitium is distorted by focal active fibroplasia and smooth muscle hyperplasia. Acute hemorrhage and finely vacuolated macrophages, which contain hemosiderin, lie within the alveolar spaces. Focally, aggregates of macrophages, multinucleated giant cells, and cholesterol clefts form compact, ill-defined nodules. Pulmonary arteries show mild medial muscle hypertrophy but no evidence of vasculitis. No viral inclusions or pneumocystis can be identified. Iron stain demonstrates hemosiderin deposition in macrophages, interstitium, and alveolar pneumocytes. Periodic acid-Schiff/diastase stain is negative in intraalveolar exudates (Fig 2).

PATIENT III. In patient II, alveolar architecture is focally effaced by a dense interstitial infiltration of lymphocytes and plasma cells with lymphoid follicles. Airspaces are dilated, and alveolar septa are lined by type II pneumocytes, with minimal fibrosis. Alveolar lumens are filled with frothy exudates, sparse neutrophils, and necrotic debris. Gomori methenamine silver stain is positive for numerous *Pneumocystis* organisms. The histopathologic findings were consistent with a diagnosis of lymphocytic interstitial pneumonitis, usually seen with active *P carinii* infection.

Immunohistochemistry

In lung biopsy specimens from both patients I and III, immunostaining for SP-A was detected along the luminal surface of the alveolar epithelium and in proteinaceous material found within the alveolar lumen, particularly in those alveoli containing increased numbers of macrophages (Fig 3, *A* and *E*). Immunostaining for mature SP-B (Fig 3, *B* and *F*) and pro-SP-B (not shown) was detected within the cytoplasm of hyperplastic/hypertrophic alveolar type II pneumocytes, as well as in proteinaceous material found within alveoli filled with macrophages. Immunostaining for pro-SP-C revealed 2 different staining patterns: (1) large dilated alveoli with very few to no pro-SP-C–positive cells and (2) smaller hyperplastic alveolar structures with an increased number of intensely stained type II pneumocytes in highly consolidated and fibrotic regions of the tissue (Fig 3, *C* and *G*). No staining for pro-SP-C was detected in luminal material.

Immunostaining for SP-D was found along the luminal surface of all alveolar structures/epithelia, as well as

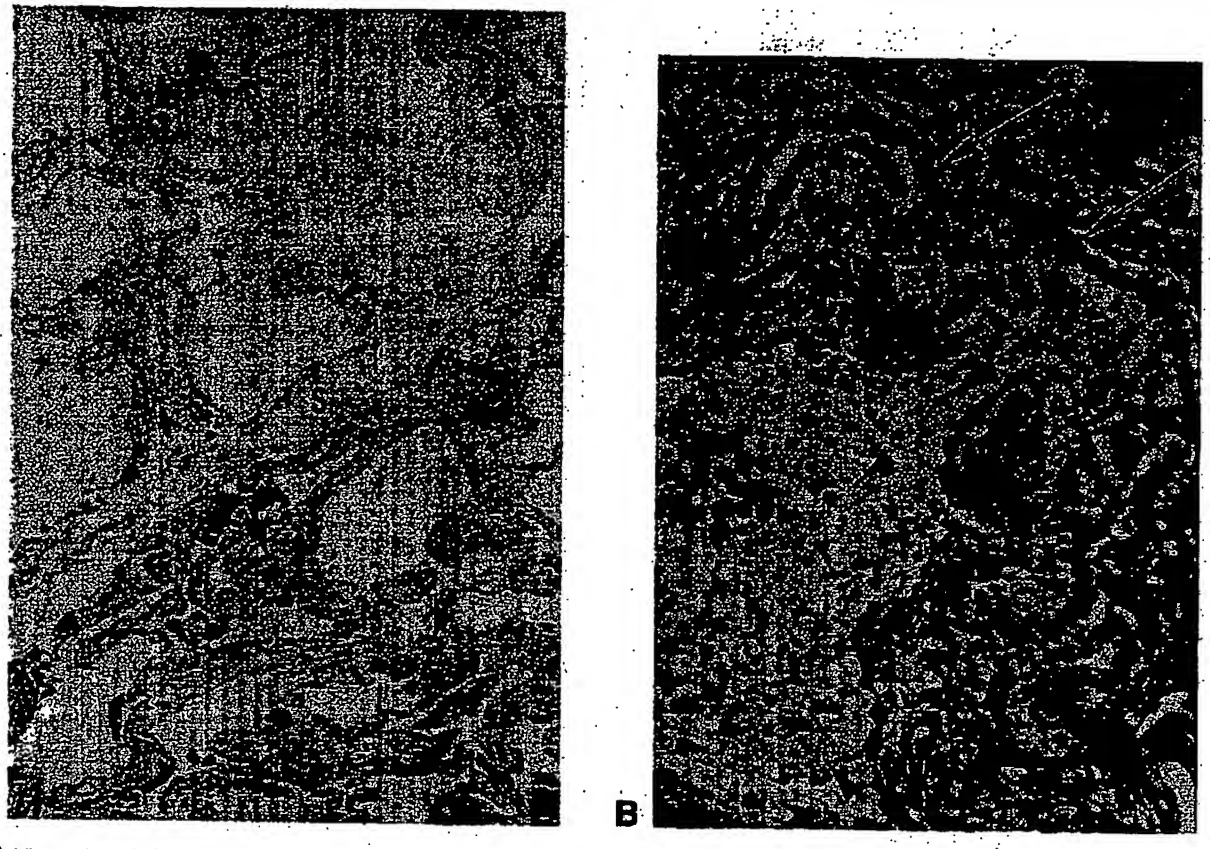

Fig 2. Histopathologic findings for patient I. Section from lung biopsy specimen obtained from patient I **(A)** shows 2 secondary lobules separated by interlobular septum *(S)*. Upper lobule shows remodeling with interstitial fibrosis and dilated airspace *(arrowheads)*. Changes in lower lobule represent active chronic interstitial pneumonia *(arrow)* (hematoxylin and eosin, original magnification ×57). Trichrome stain of lung biopsy specimen obtained from patient I **(B)** indicates advanced interstitial fibrosis in *blue*. Alveolar septa are lined by hyperplastic type II pneumocytes *(arrow)*. Airspace contains foamy macrophages *(arrowhead)* (Mason trichrome, original magnification ×227).

in the proteinaceous material found within the alveolar lumen (Fig 3, *D* and *H*). Staining of luminal material, however, varied in intensity from region to region within the tissue sections analyzed from both siblings.

In lung biopsy specimens from 13 adult patients with idiopathic pulmonary fibrosis (9 patients) and pulmonary malignancy (4 patients), immunostaining for SP-A, pro-SP-B, SP-B, pro-SP-C, and SP-D showed no evidence of decreased or absence of SPs in lung tissues (data not shown).

Immunoblot

SP-A, SP-B, and SP-D were detected in BALF from all 3 patients by Western blot analysis (data not shown). However, SP-A was less abundant in patient III than in patients I and II and the control subjects. SP-C was undetectable in BALF from all 3 patients in 2 independent assays performed with these samples but was readily detected in BALF from control groups A and B (Fig 4). Further analysis of the BALF by Western blot for pro-SP-C was not performed because, on the basis of immunohistochemistry findings, no pro-SP-C was detected in the alveolar space, and therefore it was unlikely to be retrieved in the BALF. In addition, in the absence of SP-B deficiency, pro-SP-C is not usually secreted in the alveolar space.

ELISA

SP-A levels in the BALF from patients I, II, and III were 2.09 μg/mL, 3.92 μg/mL, and 5.3 μg/mL, respectively; whereas in control subjects, SP-A levels were 22.6 ± 4.3 μg/mL ($P = .0012$) for group A and 178 ± 74 μg/mL for group B. SP-B levels in BALF from patients I, II, and III were 3 ng/mL, undetectable, and 12 ng/mL, respectively; whereas in control subjects SP-B levels were 55 ± 13.5 ng/mL ($P = .0016$) for group A and 31.7 ± 7.7 ng/mL for group B.

DNA Analysis

None of the known *SP-B* gene mutations were found in either child, and heteroduplex analysis did not suggest the possibility of novel *SP-B* gene mutations. Sequence analysis of the *SP-C* gene did not reveal any deviations from the published *SP-C* coding sequences in either child.

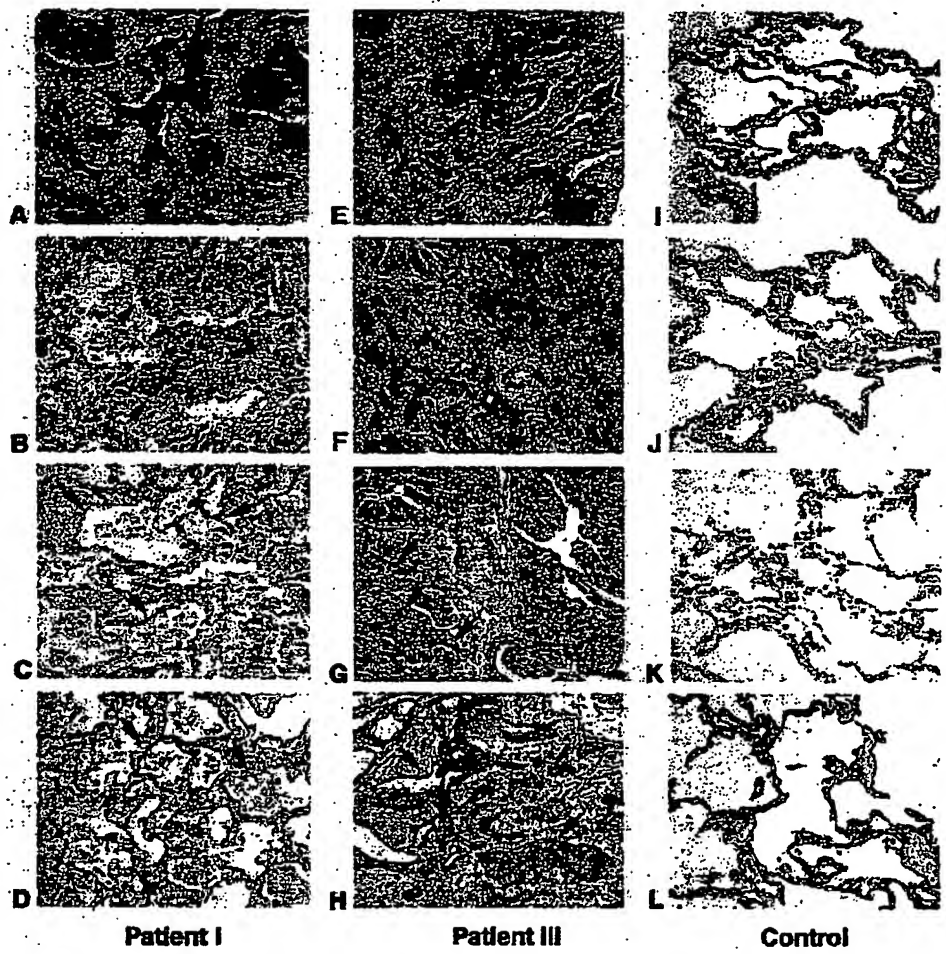

Fig 3. Immunohistochemical analysis of SP-A, SP-B, pro-SP-C, and SP-D in patients I and III. Immunohistochemical analysis of SP expression in lung biopsy specimens from patient I **(A-D)** and patient III **(E-H)** and in a normal region of lung from a control patient with focal emphysema **(I-L)**. SP-A–immunoreactive material *(arrowheads)* was detected along the alveolar epithelial cell surface and in alveoli filled with macrophages in both patient I **(A)** and patient III **(E)**. Increased numbers of SP-B–positive type II pneumocytes *(arrowheads)* were detected in alveoli of patient I **(B)** and in residual alveolar structures *(arrowheads)* of patient III **(F)**. No pro-SP-C–reactive material was detected in alveolar epithelial cells *(arrowheads)* of patient I **(C)**; and in patient III only a few, scattered, residual, alveolar structures *(arrowheads)* contained pro-SP-C-positive epithelial cells **(G)**. SP-D–immunoreactive material was detected along the apical aspect of the alveolar epithelium *(arrowheads)* in patient I **(D)** and in association with proteinaceous material *(arrowheads)* found within the alveolar lumen of patient III **(H)**. In control tissue, immunostaining for SP-A **(I)**, SP-B **(J)**, and pro-SP-C **(K)** was restricted to alveolar type II cells, whereas immunostaining for SP-D was found along the alveolar epithelial cell surface **(L)**. (Original magnification of all panels ×200).

DISCUSSION

Surfactant deficiency is the principal cause of respiratory distress syndrome seen in infants born prematurely. A selective deficiency of SP-B has also been recognized as a cause of RDS in term infants.[18] Successful trials of exogenous surfactant administration in the treatment of neonatal and adult forms of RDS demonstrate that surfactant deficiency and/or abnormality of its biophysical functions play an essential role in the development of acute lung injury.[19] SP-C associates with surfactant lipids and is a component of replacement surfactants used in the treatment of neonatal RDS. SP-C is translated as a proprotein that is synthesized exclusively by alveolar type II pneumocytes and processed intracellularly to the active SP-C protein of 33 to 34 amino acids. Mature SP-C is a hydrophobic protein that enhances the spreading and stability of surfactant phospholipids at the air-liquid interface during the respiratory cycle. In this report, we describe familial ILD with distinct histopathologic findings and with SP deficiency that consisted of absence of detectable mature SP-C in the BALF in all 3 patients. Although pro-SP-B, SP-B, SP-A, and SP-D stained strongly in lung tissues, staining for pro-SP-C was markedly decreased in type II cells, suggesting a selective deficiency in the expression or production of SP-C.

Deficiency of SP-C could be due to mutations of the *SP-C* gene, decreased

transcription, translation, processing, and secretion by alveolar type II pneumocytes or changes in catabolism of the protein. The absence of identifiable mutations in the *SP-C* gene suggests that the defect in these patients is mediated at the transcriptional or post-transcriptional level. However, a mutation in the promoter region, introns, or 3′ untranslated region that affects SP-C expression could have been missed.

Decreased transcription of the *SP-C* gene may be secondary to lung inflammation. Increased alveolar-capillary permeability and the release of various cytokines influence surfactant function and expression. Tumor necrosis factor-α, a proinflammatory cytokine, decreases SP-A, B, and C messenger RNA in vitro.[20-22] Similarly, tumor growth factor-β_1, a mediator involved in the pathogenesis of several chronic lung diseases, inhibits the expression of SP-C.[23,24] The presence of SP-C proprotein, although in markedly reduced amounts in the lung tissues, with complete absence of the mature protein in the BALF suggests that a defect in processing or secretion could also contribute to the deficiency of SP-C. The finding that immunostaining for SP-B and pro-SP-B was intense in hyperplastic alveolar type II pneumocytes suggests that these cells were preserved. It is therefore not likely that the loss of SP-C is caused by loss of alveolar type II pneumocytes.

Decreased levels of SP-A and SP-B in the BALF and apparent absence of SP-C could represent a global decrease in SP expression secondary to chronic ILD rather than a selective deficiency of SP-C. However, we found strong immunostaining for SP-A, pro-SP-B, SP-B, and SP-D along the luminal surface of the alveolar epithelium, within the cytoplasm of type II cells, and in the proteinaceous material found within the alveolar lumen. These findings suggest that a severe global deficiency of all SPs is unlikely. Because extensive remodeling has occurred, it is unclear whether sampling or tissue remodeling accounts for decreased recovery of SP-A and SP-B. These cases represent the first report of an association between SP-C and ILD. The absence of SP-C is unique to this familial form of ILD, since we have demonstrated that the expression of SP-C is preserved in other forms of non-familial pulmonary fibrosis.

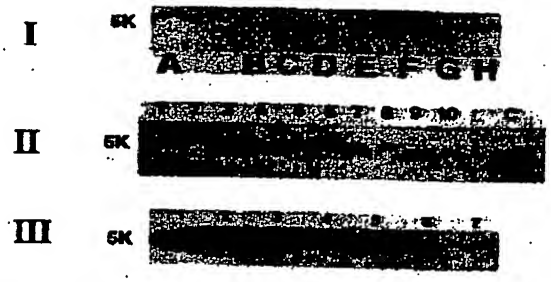

Fig 4. Western blot for SP-C. **Panel I** shows an immunoblot of BALF samples from healthy human volunteers (50 μg total protein) (*lanes A* and *E-G*) and from 3 patients (80 μg total protein) (*lanes B, C,* and *D*), with recombinant SP-C (5 ng) as control (*lane H*). SP-C is undetectable in samples from all 3 patients. **Panel II** shows BALF samples from 10 adult patients (*lanes 1-10*) with a diagnosis of idiopathic pulmonary fibrosis (50 μg total protein); SP-C levels were detectable in all patients. *C* represents recombinant SP-C. **Panel 3** shows standard for SP-C ranging from 12.5 ng in *lane 1* to 0.125 ng in *lane 7*.

The histopathologic findings described in this report may represent a distinct form of ILD. The general classification of idiopathic ILDs currently includes 4 major groups: usual, desquamative, acute, and nonspecific interstitial pneumonitis.[25,26] The histologic distinction among the different groups is based on the distribution of the histologic abnormalities, the presence of collagen-type fibrosis, honeycomb changes, the distribution of intraalveolar macrophages, and the presence of hyaline membranes.[27] The patchy distribution of the histologic abnormalities, the presence of focal areas of fibrosis and interstitial inflammation seen in the lung biopsy specimen obtained from patient I, and the presence of honeycombing on the mother's CT scan, are suggestive of a diagnosis of usual interstitial pneumonitis. However, the distribution of the disease as seen in HRCT, the overall hyperinflation of the lungs, and the lack of severe architectural distortion are atypical for the fibrosis associated with usual interstitial pneumonitis. Similarly, the presence of dilated fibrous-walled airspaces, proteinaceous material rich in SPs in the alveolar space, collections of foamy macrophages, cholesterol granulomas, and evidence of hemosiderosis make the histopathologic findings[27] in this case distinct from the typical picture of usual interstitial pneumonitis. The presence of hemosiderosis in this case could not be explained by the presence of vasculitis.

Although lung diseases secondary to GER may exhibit similar pathology, the clinical course seen with these patients is not consistent with a diagnosis of recurrent aspiration secondary to GER.[28] Specifically, patient III had 2 surgical fundoplications for the treatment of GER but continued to show progressive lung disease and have lipid-laden macrophages in the BALF after the 2 surgeries led to complete resolution of GER symptoms. Persistence of lipid-laden macrophages in the BALF after successful treatment of GER suggests that endogenous lipids are the primary source for intra-alveolar and interstitial accumulation of lipids.

In summary, we have identified 3 family members with ILD who presented with similar clinical courses and radiographic and histopathologic features and were found to have low levels of SP-A and SP-B in the BALF and an apparent absence of the mature form of SP-C. This report suggests

that ILD that consists of pulmonary fibrosis and emphysema may be associated with SP-C deficiency.

We thank Sherri A. Profitt for her technical assistance in performing the immunohistochemistry.

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Attachment 2

Heterozyg sity for a Surfactant Protein C Gene Mutation Ass ciated with Usual Interstitial Pneumonitis and Cellular Nonspecific Interstitial Pneumonitis in One Kindred

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Familial pulmonary fibrosis is a heterogeneous group of interstitial lung diseases of unknown cause that is associated with multiple path logic subsets. Mutations in the surfactant protein C (SP-C) gene (*SFTPC*) are associated with familial desquamative and nonspecific interstitial pneumonitis. Genetic studies in familial usual interstitial pneumonitis have been inconclusive. Using a candidate g ne approach, we found a heterozygous exon 5 + 128 T→A transversion of *SFTPC* in a large familial pulmonary fibrosis kindred, including adults with usual interstitial pneumonitis and children with cellular nonspecific interstitial pneumonitis. The mutation is predicted to substitute a glutamine for a conserved leucine residue and may hinder processing of SP-C precursor protein. SP-C precursor protein displayed aberrant subcellular localization by immunostaining. Electron microscopy of affected lung revealed alve lar type II cell atypia, with numerous abnormal lamellar bodies. M use lung epithelial cells transfected with the *SFTPC* mutation wer notable for similar electron microscopy findings and for exaggerated cellular toxicity. We show that an *SFTPC* mutation segregates with the pulmonary fibrosis phenotype in this kindred and may cause type II cellular injury. The presence of two different pathologic diagnoses in affected relatives sharing this mutation indicates that in this kindred, these diseases may represent pleiotropic manifestations of the same central pathogenesis.

Keyw rds: pulmonary fibrosis; familial; surfactant protein C; interstitial lung disease; genetics

The familial forms of pulmonary fibrosis (FPF) are described as the occurrence of interstitial pneumonitis in at least two members of a family. It is unknown what proportion of idiopathic pulmonary fibrosis (IPF) is familial, but it is estimated that 0.5 to 2.2% of cases have a genetic basis (1). To date, over 68 kindreds presumed to have IPF have been reported (1–3). Diagnostic heterogeneity cannot be excluded in a number of these reports, however, as many of these families lack surgical lung biopsy specimens. Marshall reviewed the clinical and epidemiologic findings of 67 patients from 25 families with IPF, from which 32% of cases had surgical lung biopsies performed. Clinical signs/symptoms, treatment outcomes, and histologic findings (usual interstitial pneumonitis [UIP], the pathologic correlate of IPF) were indistinguishable from sporadic IPF, except that cases were younger at diagnosis (1). Familial forms of desquamative interstitial pneumonitis (4) and lymphocytic interstitial pneumonitis have also been described (5). Several FPF kindreds have been reported that include affected adults and children (6–9). The familial form of IPF/UIP is likely transmitted as an autosomal dominant trait with reduced penetrance (1–3, 10, 11). Genetic studies analyzing candidate loci near the human leukocyte antigen region of chromosome 6 in familial IPF/UIP (9) and in sporadic pulmonary fibrosis have been largely inconclusive (12, 13). Polymorphisms in the genes encoding interleukin-1 receptor antagonist (14), tumor necrosis factor-α (14), and angiotensin-converting enzyme (15) have been suggested to play a role in sporadic forms of pulmonary fibrosis.

Recently, a mutation in the gene (*SFTPC*) encoding the hydrophobic, lung-specific surfactant protein C (SP-C) was discovered in association with an infant and mother with cellular nonspecific interstitial pneumonitis (NSIP) and desquamative interstitial pneumonitis, respectively (16). A heterozygous G to A transition was identified at the first base of intron 4 (IVS4+1 G→A) of both patients' DNA that abolished the normal IVS4 5′ splice site, resulting in a deletion of 37 amino acids from the carboxy-terminal (C-terminal) region of *SFTPC*.

We report here our study of *SFTPC* in a large FPF kindred, including 14 affected members spanning 6 decades. The clinical findings of three members of this family have been previously described (6, 17).

METHODS

FPF Kindred/Specimens

The Vanderbilt University Institutional Review Board approved this investigation. This kindred (Figure 1) contains 97 members, including 11 adults and 3 children with pulmonary fibrosis. Six adults and three children have pathologic diagnoses of UIP and cellular NSIP, respectively. Clinical records of affected members were reviewed (Table 1). Autopsy, explant, or surgical biopsy of paraffin-embedded lung tissue was reviewed by a lung pathologist (Figure 2).

Blood and paraffin-embedded lung tissue was used for isolation of DNA using a PureGene Kit (Gentra Systems, Minneapolis, MN). Control DNA was the Ethnic Diversity Set (DNA Polymorphism Discovery Resource; Coriell Cell Repositories, Camden, NJ). Control lung tissue was resected for malignancy.

Linkage/Mutational Analysis

A microsatellite marker within 9 kb of *SFTPC* was used for linkage analysis. Primers (fluorescently labeled) used for polymerase chain reaction of the marker have been described (18). Alleles were analyzed

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The URL for data in this article is http://www.ncbi.nlm.nih.gov/genbank/, and accession numbers for the *SFTPC* sequence and the microsatellite marker are J03890 and L16861, respectively.

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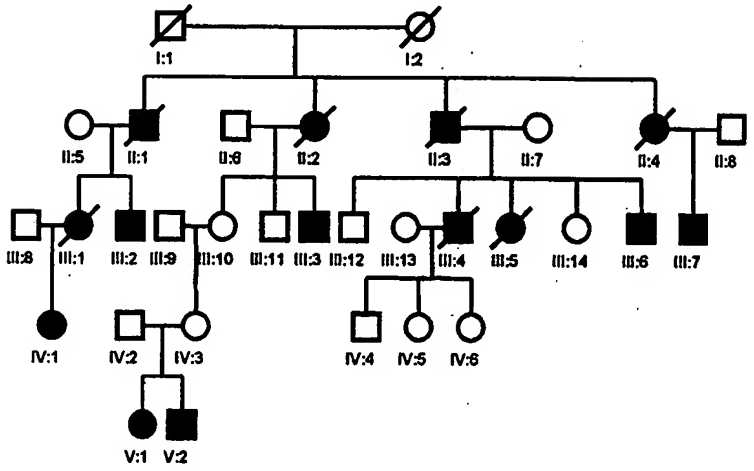


Figure 1. Condensed pedigree of kindred. Generation (I–V): identifier number is listed below each family member (*circle* = female, *square* = male, *open symbol* = unaffected, *closed circle* = affected, *slash through symbol* = deceased).

on an automated gene sequencer (Applied Biosystems, Foster City, CA). A logarithm of the odds score was calculated assuming autosomal dominant inheritance of a gene with a disease allele frequency of 0.0001. The marker variant was assumed to have an equally rare allele frequency. Using the program VITESSE (19), analysis was done assuming that apparently unaffected individuals were not disease gene carriers, using phenotype information on only affected individuals.

For mutational analysis, polymerase chain reaction primers (Integrated DNA Technologies, Coralville, IA) used to amplify *SFTPC* have been described (20) (Figure 3). Amplicons were used as templates for dideoxy fingerprinting and restriction analysis. *SFTPC* was screened by dideoxy fingerprinting using a primer scanning approach (Figure 3) with variants confirmed by dideoxy sequencing using the Thermo Sequenase Cycle Sequencing Kit (Amersham Pharmacia Biotech, Piscataway, NJ).

Using a *Bsr*1 restriction site created by the mutation, the presence or absence of the exon 5 + 128 T→A mutation was screened from DNA of available family members and 88 control chromosomes. A 549 base pair (bp) exon 5 polymerase chain reaction amplicon was assessed for the presence of a *Bsr*1 site with restriction products (New England Biolabs, Beverly, MA) resolved on a 2% metaphor agarose gel.

Immunohistochemistry

Lung tissue sections (3–5 μm) from patient III:3 were deparafinized in xylene and hydrated through graded ethanols. Using the Ventana ES (Ventana Medical Systems, Tucson, AZ), immunostaining was performed using diluted rabbit polyclonal antibody to SP-C precursor protein (proSP-C). Avidin-biotin staining with diaminobenzidine as the chromogen and counterstaining with hematoxylin were performed.

Plasmid Constructs, Cell Transfection, and Toxicity Assay

Normal full-length *SFTPC* was amplified from unaffected family member DNA. pGEMTeasy was transformed with this amplicon. Inserts were excised and ligated into pCINeo using *Not1*, *Nhe1*, and *Spe1* sites. This plasmid/insert was used to create the mutation by site-directed mutagenesis using the Quickchange kit (Stratagene, La Jolla, CA) and mutation-overlapping primers (Figure 3). Mouse lung epithelial (MLE12) cells were transfected with plasmids containing normal and mutant *SFTPC*, using FuGENE6 (Roche, Indianapolis, IN). Pooled stable lines were grown in Hite's media under G418 selection (American Type Culture Collection, Manassas, VA). Supernatants and lysates of 10^5 viable nontransfected, wild-type, or mutant *SFTPC*-transfected MLE cells incubated for 24 hours were assayed for cellular toxicity using the lactate dehydrogenase (LDH)-based CytoTox96 Assay (Promega, Madison, WI). Statistical significance was tested with the unpaired Student's *t* test.

Electron Microscopy

Electron microscopy (EM) methods have been described (21). Briefly, lung tissue was fixed in Karnovsky's solution and then postfixed and

TABLE 1. CLINICAL AND PATHOLOGIC DATA FROM AFFECTED INDIVIDUALS

Patient	Sex	Age at Dx	Year of Dx	Year of Death	Clinical and Pathologic Descriptors
II:1	M	29	1942	1952	Dyspnea, cough, clubbing; CXR with "diffuse granular infiltration"; path report "fibrocystic pulmonary dysplasia."
II:2	F	57	1976	1986	Dyspnea, cough; cause of death "pulmonary fibrosis."
II:3	M	20	1945	1990	Dyspnea, cough, clubbing; CXR with "bilateral interstitial fibrosis"; TLC 52%, D_{LCO} 51%.
II:4	F	41	1967	1986	Dyspnea, clubbing; cause of death "pulmonary fibrosis."
III:1	F	17	1959	1964	Dyspnea, cough, clubbing; CXR "extensive, scattered nodular infiltrations throughout both lungs"; FVC 21%; Asian influenza pneumonia before onset; path report "interstitial pulmonary fibrosis (Hamman-Rich Disease)."
III:2	M	20	1965	1991	Dyspnea, cough; path report UIP.
III:3	M	32	1986	Alive	Dyspnea; CXR "diffuse coarse reticulation with multiple lucencies"; DLT 2000; path UIP by slide review.
III:4	M	34	1989	2000	Dyspnea, cough, clubbing; TLC 60%, D_{LCO} 41%; CXR "diffuse interstitial infiltrates, worse at bases"; DLT 2000; path UIP by slide review.
III:5	F	6 mo	1952	1953	Failure to thrive, cough, cyanosis; CXR "ground glass appearance with fine fibrillary infiltration through both lungs"; path NSIP by slide review.
III:6	M	40	2001	Alive	Pulmonary fibrosis diagnosed as a child; mild dyspnea in adulthood; age 40 CXR "worsening reticulonodular interstitial infiltrates."
III:7	M	44	1990	Alive	Diagnosed as "environmental lung scarring"; CXR "bilateral scarring."
IV:1	F	37	1999	Alive	Dyspnea, cough; TLC 70%, D_{LCO} 60%; chest CT "bilateral patchy interstitial opacities and honeycombing"; path report UIP.
V:1	F	17 mo	1997	Alive	Respiratory failure; RSV pneumonia before disease onset; path report NSIP.
V:2	M	4 mo	1998	Alive	Respiratory failure; influenza B pneumonia before disease onset; path report NSIP.

Definition of abbreviations: CT = computed tomography; CXR = chest X-ray; DLCO = diffusion capacity of carbon monoxide (% predicted); DLT = double lung transplantation; Dx = diagnosis; FVC: = forced vital capacity (% predicted); N/A = not available; NSIP = nonspecific interstitial pneumonitis; Path = pathology; RSV = respiratory syncytial virus; TLC = total lung capacity (% predicted); UIP = usual interstitial pneumonitis.

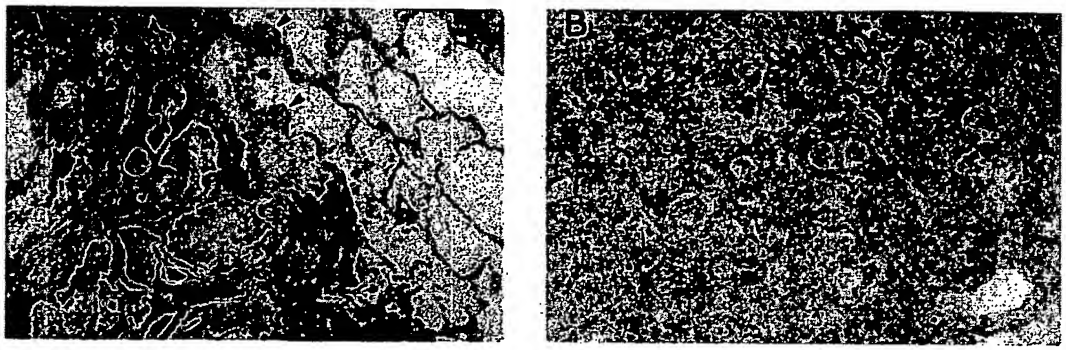


Figure 2. Photomicrographs of representative lung tissue. (*A*) UIP. This low power view of patient III:3's lung biopsy shows coarse scarring with airspace dilatation and metaplastic cuboidal to columnar epithelium (*left*), normal alveolar tissue (*right*), and two fibroblast foci at the interface (*arrowheads*; hematoxylin and eosin ×12.5). (*B*) Cellular NSIP. Autopsy lung tissue from patient III:5 shows homogenous alveolar septal thickening by a cellular infiltrate and intra-alveolar cells and granular material (hematoxylin and eosin ×12.5).

dehydrated. Embedded sections were examined by light microscopy. Thin sections were cut and mounted on grids that were observed on a Phillips 300 electron microscope.

RESULTS

Linkage and Mutational Analysis

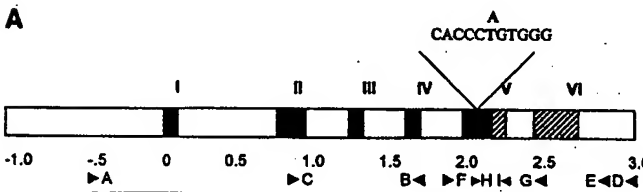
Under the assumed autosomal dominant inheritance, a logarithm of the odds score of 4.33 at a recombination fraction of 0.00 was generated between the FPF phenotype and the marker. An abnormal dideoxy-fingerprinting pattern was obtained from polymerase chain reaction fragments containing exon 5 from DNAs of three affected family members (data not shown). Sequencing of these three affected member's DNA revealed a heterozygous exon 5 + 128 T→A transversion (Figure 4) that substitutes a glutamine for leucine at the highly conserved amino acid position 188 of the C-terminal region of proSP-C. This region is crucial to proper intracellular trafficking/folding of proSP-C (22–24). Restriction patterns of DNAs from six affected and two obligate heterozygous unaffected family members indicated heterozygosity for the mutation (Figure 5 and data not shown). The mutation was not present in DNAs from four unaffected family members and 88 control chromosomes.

Immunohistochemistry

In lung from patient III:3, immunostaining for proSP-C was evident without antigen retrieval within rows of abnormal type II cells lining thickened alveoli and terminal airways. Large, dysplastic, cuboidal type II cells were seen proliferating throughout affected areas of lung. Staining for proSP-C revealed diffuse cytoplasmic distribution in type II cells. In contrast, in control type II cells, proSP-C was localized adjacent to lamellar bodies (Figure 6).

Electron Microscopy

Lung tissue from two affected family members was notable for dense fibrosis and distorted cellular architecture. Dysplastic type II cells contained many abnormal-appearing lamellar bod-



B

Primer	Sequence 5' – 3'
A	TGGAACTGGTCCTTGCAGGGAA
B	CTGGCTTGTACGCGATCAGC
C	CATGACCTCATGCCTGTCTC
D	AAAGCCCGTGGATGCTCCCT
E	ACAGAGGGCGAATGGAAA
F	GACTGCCCCTCTCCTATTCC
G	GTGGGAAGTACCGGTCTGTG
H	GGCATGGCCGTGAACACCCAG
	TGTGGCGAGGTGCC
I	GGCACCTCGCCACACTGGGTG
	TTCACGGCCATGCC

C

Primer	Sequence 5' – 3'
1	CCTTGGCCACCTGAAAGTT
2	CCAGGAACAAACAGGCTTCA
3	CTATCCTCCCTGGCCTGTTT
4	TGGAGCCAGAATAAGGAGGA
5	TCTGATCTCCTCAGCCCTTC
6	CTCATCGTCGTGGTGATTGT
7	CAGCTCAGGCTTTTCCACA
8	CTGGTTACCACTGCCACCTT
9	TGATCTCCAGCATTCTGTGC
10	GACTGCCCCTCTCCTATTCC
11	CCGCAGTGCCTACGTCTAAG
12	GGTCATCCAGGCAACTCG
13	ATGGGAGTGGGCAGAGGT
14	CCCTGTGGCTTTCCTTTTTC
15	ATCCCACACCTCTCACCATC

Figure 3. Diagram of *SFTPC* gene. (*A*) Introns (*open bars*), exons I–V (*closed bars*), and untranslated exons V–VI (*cross-hatched bars*) are shown. Scale below gene is in kilobases. Approximate location of exon 5 + 128 T→A mutation is shown above exon 5. Location of polymerase chain reaction primers (A–I, 1–15) is shown below gene. The *right arrow* represents forward primer, and the *left arrow* represents reverse primer (*A* and *B* = first segment, *C* and *D* = second segment, *C* and *E* = nested second segment, *F* and *G* = restriction analysis segment, *H* and *I* = mutation site direct primers). (*B*) Sequence f *SFTPC* primers A–G. (*C*) Sequence of *SFTPC* dideoxy-fingerprinting primers 1–15.

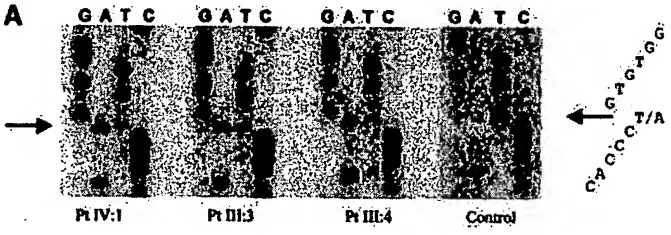


Figure 4. Sequencing gel of three affected patients and one control subject. Numbers below gel represent patient identifiers shown in Figure 1. (*A*) Heterozygous T→A mutation is evident at genomic position exon 5 + 128 in three patients (*arrows*). Sequence of segment is shown to right of gel. (*B*) Amino acid sequence homology of carboxy terminal region of SFTPC containing leucine at position 188 (*bold text*), which is highly conserved across species (*underlined*).

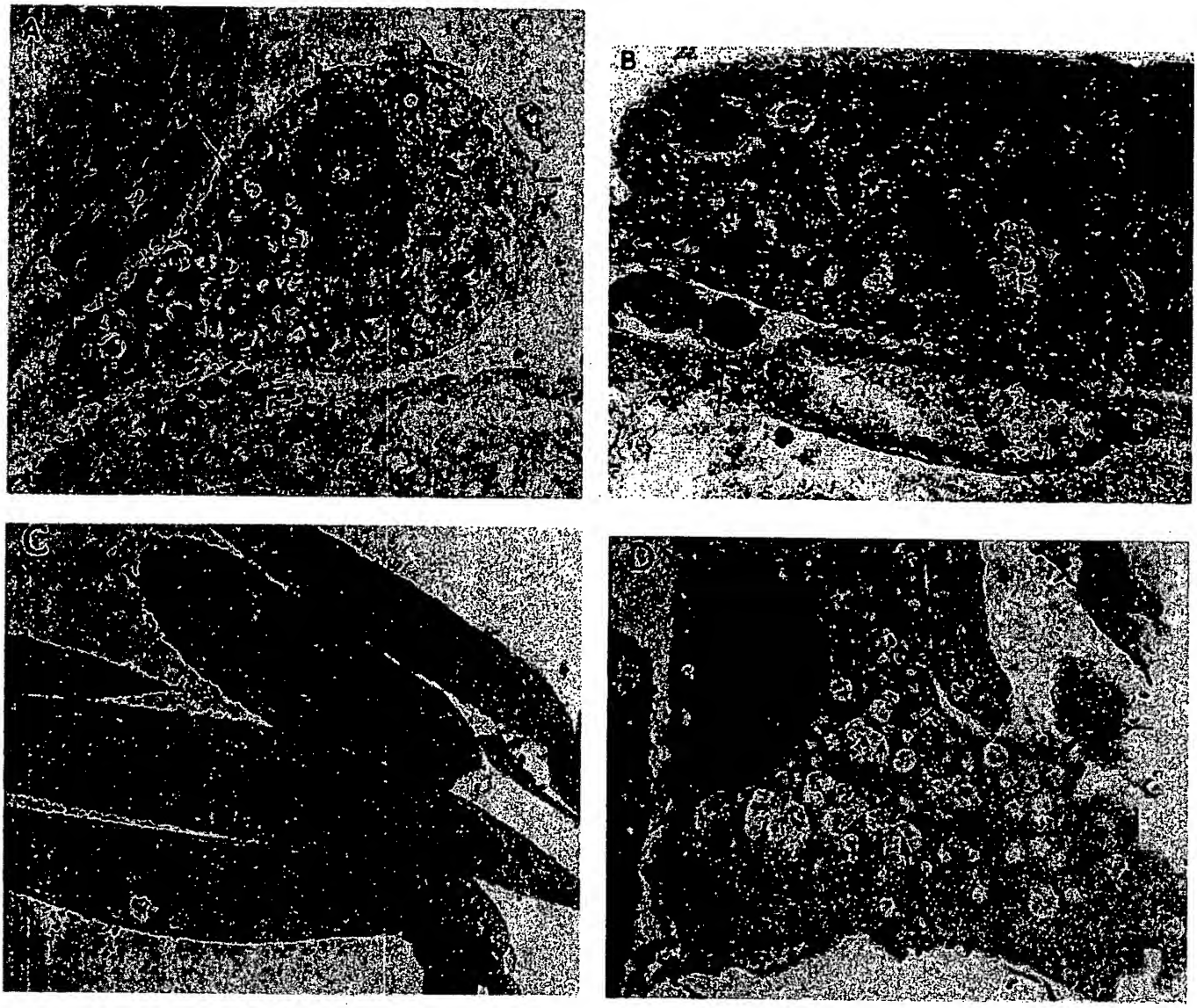


Figure 7. Electron microscopy images. (*A*) Lung from FPF patient IV:1. Type II cell (*center*) shows abundant, atypical lamellar bodies (*arrow*) accumulating in the cytoplasm. A loss of basement membrane and cell–cell contact (*bottom*) is present as type II cell sloughs into airway filled with amorphous debris (*top, right*). Dense fibrosis is evident (*left*) (×8,000). (*B*) Type II cell from FPF patient IV:1 shows cytoplasmic electron-dense dark bodies (*arrows*) (×40,000). (*C*) MLE cells transfected with normal *SFTPC* (×5,600). (*D*) MLE cell transfected with *SFTPC* containing the exon 5 + 128 T→A transversion. Excessive inclusions resembling lamellar bodies are evident accumulating in the cytoplasm (*arrows*) (×4,400).

lacks the C-terminal 22 amino acids, proSP-C is restricted to the endoplasmic reticulum (23). Likewise, in A549 cells transfected with *SFTPC* deletional mutants whose products lack the C-terminal 10 amino acids of proSP-C, the proprotein remains localized in the endoplasmic reticulum without proper proteolysis (22). The conserved L188 residue that is altered by the mutation found in this kindred lies within this critical region. In addition, in studies using similar *in vitro* constructs containing a rat proSP-C C-terminal point mutation (C186G) of a conserved cysteine residue corresponding to the human proSP-C residue (C189) adjacent to L188, proSP-C is not processed correctly and is retained in early secretory compartments associated with misfolded protein aggregates (24).

There is increasing evidence that implicates SP-C in the pathophysiology of some types of chronic lung disease. Intracellular accumulation of incompletely processed proSP-C has been demonstrated in SP-B–deficient infants with congenital alveolar proteinosis (27). Multiple heterozygous mutations in *SFTPC* have been reported in association with children suffering from interstitial lung disease (16, 28), including familial and sporadic occurrences. Most of these cases likely represent desquamative interstitial pneumonitis or NSIP. Similar to this report, many of these mutations occur within the C terminus of proSP-C, including a patient with a mutation encoding substitution (L188R) of the same conserved leucine residue that is altered in our kindred (L188Q). In addition, a deficiency of SP-C has been described in a small kindred suffering from a poorly defined form of interstitial pneumonitis, despite no sequence variation in *SFTPC* (29). To our knowledge, the *SFTPC* exon 5 + 128 T→A transversion that we describe in this kindred is the first description of an SP mutation associated with UIP.

Therefore, we propose the L188Q mutation may cause misfolding and trapping of proSP-C in or near the endoplasmic reticulum without delivery to distal secretory compartments. This is supported by immunohistochemistry, as unlike control lung tissue, proSP-C did not localize adjacent to type II cell lamellar bodies in diseased lung tissue but was found diffusely throughout the cytoplasm (Figure 6). The many abnormal in-

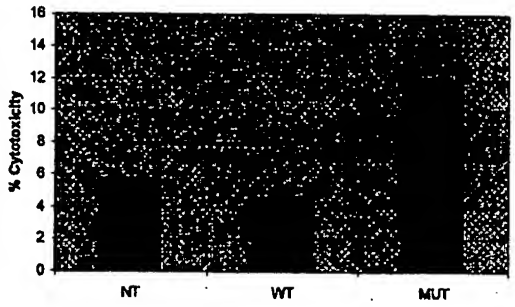


Figure 8. MLE cytotoxicity assay. Cell lines are shown on *x* axis (NT = n ntransfected MLE cells, WT = normal *SFTPC*-transfected MLE cells, MUT = exon 5 + 128 T→A *SFTPC*-transfected MLE cells). The percentage of cytotoxicity = LDH activity of cell supernatant/LDH activity of cell lysate. Data shown represents average of eight experiments, with standard deviation represented by *error bars*. *$p < 0.05$ for comparison between MUT-NT and between MUT-WT.

clusions resembling lamellar bodies seen by EM of affected lung tissue and mutant *SFTPC*-transfected MLE cells could possibly be secondary to this abnormal processing of proSP-C. Although the identity of the dark bodies seen by EM accumulating in the cytoplasm of affected lung type II cells is unclear, electron-dense inclusions possibly representing early multivesicular bodies have been described in EM studies of infants with respiratory distress and SP-B deficiency (30), SP-B −/− deficient mice with unprocessed proSP-C (21) and in *in vitro* transfection studies with C-terminal proSP-C point mutants associated with misfolded protein aggregates (24).

The mutation we report has the potential to cause alveolar type II cellular injury, as transfection of MLE cells with *SFTPC* differing in only one single bp (exon 5 + 128 T→A) results in a threefold increase in cytotoxicity compared with MLE cells transfected with the normal gene. On the EM of affected lung tissue, type II cellular injury is suggested, as many cells lack basement membranes and lose structural integrity or cell–cell contact. Such morphologic findings have been described in early type II cellular death or apoptosis (31, 32). Alveolar type II cell injury and altered re-epithelialization associated with an excessive fibrotic phenotype has been implicated in pulmonary fibrosis (33, 34). Although the exact mechanism of alveolar epithelial injury associated with this *SFTPC* mutation will likely require animal models to be elucidated, accumulation of misfolded proSP-C could eventually lead to type II cellular damage. Similar pathophysiology is seen in forms of α1-antitrypsin deficiency, where the Z mutant protein is misfolded, leading to hepatocyte injury and cirrhosis (35). The list of genetic disorders associated with protein misfolding is growing, including autosomal dominant diseases such as congenital nephrogenic diabetes insipidus, protein C deficiency, and hereditary blindness (36).

The presence of the exon 5 + 128 T→A mutation on one allele suggests that it may have a dominant negative effect on the function or processing of SP-C. SP-C can form oligomers and interacts with surfactant protein B (37). Oligomeric sorting in the trafficking of proSP-C was recently shown to occur *in vitro*, as proSP-C was retained in early secretory compartments in deletional mutants lacking endogenous targeting signals, but the same cells cotransfected with full length proSP-C were capable of secretion to late compartments (38). Thus, the abnormal protein theoretically could hinder trafficking of normal proSP-C or possibly surfactant protein B. Also, a deficiency in mature secreted SP-C may be contributing to the pathophysiology of this disease as well. This is supported by the observation that SP-C −/− mice have normal lung function at birth but survive into adulthood with abnormal surfactant that is unstable at low lung volumes (39). Deficiency of SP-C could theoretically promote alveolar instability, leading to lung inflammation and injury.

Interestingly, two different subsets of pulmonary fibrosis were found by pathologic examination to exist in members of our family who share a mutation in *SFTPC*. We cannot exclude the possibility of cellular NSIP occurring as a precursor lesion to UIP in this family. Although some investigators believe that predominately inflammatory subsets of pulmonary fibrosis such as desquamative interstitial pneumonitis are early stages of UIP (40, 41), current evidence suggests they are separate pathologic and clinical entities (42). More likely, cellular NSIP and UIP are pleiotropic effects of the genetic defect that are occurring in our kindred. It is possible that the forms of FPF differ on the molecular level from sporadic pulmonary fibrosis, resulting in greater diversity in clinical and pathologic features. This is evident in this study, where some patients with a pathologic diagnosis of UIP had disease onset in childhood or early adulthood with long durations of illness, in contrast to the more aggressive course of sporadic IPF with later disease onset. The genetic defect in this family does not display complete penetrance, as two unaffected (to date) family members heterozygous for the mutation were found in this kindred. Reduced penetrance in familial IPF has similarly been reported by multiple authors (1–3, 10, 11). Thus, secondary modifiers likely affect the penetrance of this *SFTPC* mutation. Although the role of environmental influence on *SFTPC* mutations has not been studied, triggers such as infection or toxins may influence the wide diversity in clinical presentation of *SFTPC*-associated pulmonary fibrosis. Respiratory viral infections were temporally related to onset of disease in at least three members of this family. Multiple viral agents have been suggested to play a role in pulmonary fibrosis, including Epstein-Barr virus, influenza, and hepatitis C (43). Unlike surfactant proteins A and D, no antiviral or immune properties have been associated with SP-C (25).

In conclusion, we report a mutation in *SFTPC* associated with a large FPF kindred, including adults with the most common pathologic subset of pulmonary fibrosis, UIP, and children with cellular NSIP. The mutation *in vitro* is associated with cellular toxicity; thus, type II cell damage may underlie the pathogenesis of pulmonary fibrosis associated with this mutation. Our results suggest that within this kindred, different pathologic forms of FPF may represent pleiotropic manifestations of the same *SFTPC* mutation.

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Mutations in the Surfactant Protein C Gene Associated With Interstitial Lung Disease*

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(CHEST 2002; 121:20S–21S)

Abbreviations: ILD = interstitial lung disease; SP-C = surfactant protein C

The molecular basis for most forms of interstitial lung disease (ILD) is unknown. We recently identified a splicing mutation (c.460 + 1G > A) in the gene encoding surfactant protein C (SP-C) in two affected individuals with familial ILD.[1] To test the hypothesis that SP-C mutations cause both sporadic and familial lung disease, we analyzed the SP-C gene sequence in infants with chronic lung diseases of unknown etiology. Mutations on one allele of the SP-C gene were identified in 11 of 34 patients evaluated. One infant had a mutation in the same location, but with a different nucleotide substitution (G > T) as the patients with the c.460 + 1G > A mutation. The clinical, histopathologic, and biochemical findings of these patients were similar, and included skipping of exon 4, expression of an aberrantly migrating proprotein corresponding to the size of the deletion, and reduced amounts of normal pro-SP-C. However, neither parent had a history of lung disease or carried the mutation. The occurrence of a *de novo* mutation functionally

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identical to a familial mutation in infants with the same phenotype strongly supports the hypothesis that the mutations were causally related to their lung disease. Missense SP-C mutations that resulted in amino-acid substitutions in residues highly conserved across species (P30 L, I73T, G100V, Y104H, P115 L, I126R, T187N, and L188R), as well as a frameshift mutation (140delA) associated with expression of a stable transcript, were identified in 10 other infants, 6 of whom had a family history of lung disease. None of the identified mutations were found on 100 control chromosomes, indicating that they are not common polymorphisms. We conclude that mutations in the SP-C gene are a cause of both familial and sporadic ILD. While the pathophysiologic mechanisms remain to be elucidated, the finding of mutations on one allele suggests a dominant negative effect on SP-C or pro-SP-C function or metabolism.

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Mapping Susceptibility Genes for the Induction of Pulmonary Fibrosis in Mice*

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(CHEST 2002; 121:21S)

Abbreviations: FGF = fibroblast growth factor; QTL = quantitative trait locus; TNF = tumor necrosis factor

Pulmonary fibrosis is a potentially fatal disease that can result from radiation or chemotherapeutic treatment of malignancy, exposure to certain irritants, and idiopathic events. Our studies have focused on the genetic mechanisms underlying this disease through the analysis of inbred mouse strain variation in susceptibility to fibrosis induction.. We have identified an inbred mouse strain (DBA/2) that is highly susceptible to bleomycin-induced pulmonary fibrosis and is genetically very dissimilar to the standard fibrosis-sensitive strain, C57BL/6, but similar to the standard fibrosis-resistant strain, BALB/c. Analysis of a set of backcross progeny generated between DBA/2 and BALB/c strains indicates that susceptibility to development of pulmonary fibrosis is controlled primarily by a few (two to three) independent genetic loci. Genetic linkage using quantitative trait locus (QTL) analysis has led to the chromosomal assignment of two of these susceptibility loci. One susceptibility gene is located within a subregion of chromosome 6 that contains a cluster of genes that are members of the tumor necrosis factor (TNF)-receptor family, including the 55-kd TNF-α_1 receptor. The second susceptibility gene has been mapped to the telomeric end of chromosome 13, within an interval encompassing fibroblast growth factor (FGF)-10, a member of the FGF gene family that is expressed predominantly in the developing lung. Analysis of allelic variation in these candidate genes is underway in order to evaluate their utility as genetic markers for fibrosis susceptibility and to elucidate their possible role in influencing the disease process.

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Uses of Expression Microarrays in Studies of Pulmonary Fibrosis, Asthma, Acute Lung Injury, and Emphysema*

Roger S. Mitchell Lecture

Dean Sheppard, MD

Expression microarrays are a powerful tool that could provide new information about the molecular pathways regulating common lung diseases. To exemplify how this tool can be useful, selected examples of informative experiments are reviewed. In studies relevant to asthma, the cytokine interleukin-13 has been shown to produce many of the phenotypic features of this disease, but the cellular targets in the airways and the molecular pathways activated are largely unknown. We have used microarrays to begin to dissect the different transcriptional responses of primary lung cells to this cytokine. In experiments designed to identify global transcriptional programs responsible for regulating lung inflammation and pulmonary fibrosis, we performed microarray experiments on lung tissue from wild-type mice and mice lacking a member of the integrin family know to be involved in activation of latent transforming growth factor (TGF)-β. In addition to identifying distinct cluster of genes involved in each of these processes, these studies led to the identification of novel pathways by which TGF-β can regulate acute lung injury and emphysema. Together, these examples demonstrate how careful

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Attachment 4

Review

Surfactant gene polymorphisms and interstitial lung diseases

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Abstract

Pulmonary surfactant is a complex mixture of phospholipids and proteins, which is present in the alveolar lining fluid and is essential for normal lung function. Alterations in surfactant composition have been reported in several interstitial lung diseases (ILDs). Furthermore, a mutation in the surfactant protein C gene that results in complete absence of the protein has been shown to be associated with familial ILD. The role of surfactant in lung disease is therefore drawing increasing attention following the elucidation of the genetic basis underlying its surface expression and the proof of surfactant abnormalities in ILD.

Keywords: genetics, interstitial lung disease, polymorphism, surfactant

Introduction

The role of surfactant in interstitial lung disease (ILD) has drawn increasing attention in recent years, particularly with the publication of the genetic determinants of surfactant expression. This review will focus on surfactant abnormalities in ILD, with emphasis on surfactant protein (SP) gene polymorphisms.

Pulmonary surfactant is a mixture of phospholipids and surfactant-specific proteins, which are essential for normal lung function. The main function of pulmonary surfactant is to stabilize the alveoli throughout the respiratory cycle, preventing alveolar collapse at the end of expiration. Surfactant-specific proteins are also involved in host defense and inflammatory processes in the lung.

Between 90% and 95% of lung surfactant is made up of lipids, with the remainder being proteins. Roughly 65% of the lipid component of surfactant is phosphatidylcholine. The remaining 30–35% consists predominantly of phosphatidylglycerol, while phosphatidylinositol, phosphatidylethanolamine, phosphatidylserine and sphingomyelin are also present in small amounts. The surfactant-specific proteins are mainly composed of four surfactant-associated proteins: SP-A, SP-B, SP-C and SP-D, along with a minor component of mainly serum-derived proteins. SP-A and SP-D are hydrophilic, while SP-B and SP-C are highly hydrophobic proteins.

Surfactant phospholipids and SP-C are synthesized only in the type II alveolar epithelial cells. The proteins SP-A, SP-B and SP-D are produced by Clara cells and type II alveolar epithelial cells in the lung. SP-A is the most abundant surfactant protein and is completely lipid-bound. It is a multimer containing the product of two genes: SP-A1 and SP-A2. SP-A and SP-D proteins are structurally similar collagenous glycoproteins belonging to the collectin superfamily. They are involved in host defense and recognize the carbohydrate moiety on the surface of pathogens. SP-B and SP-C are crucial in reducing surface

aa = amino acid; BAL = bronchoalveolar lavage; IPF = idiopathic pulmonary fibrosis; ILD = interstitial lung disease; SNP = single nucleotide polymorphism; SP = surfactant protein.

tension by nhancing the adsorption and spreading of phospholipid at the air–liquid interface. Absence of SP-B production is lethal in infants and experimental animals [1].

Surfactant in interstitial lung disease

Several studies have explored the surfactant levels in bronchoalveolar lavage (BAL) fluid in patients with ILDs. More recently, serum levels of SPs have been studied and correlated with disease progression.

Bronchoalveolar lavage studies

Idiopathic pulmonary fibrosis (IPF)

Early studies of surfactant in the bleomycin animal model of lung injury showed significant alterations in the composition and biophysical properties of surfactant. Studies of phospholipid content of surfactant in BAL from IPF patients showed significant abnormalities, including reduced alveolar phospholipid, decreased content of phosphatidylglycerol and a reduction in the phosphatidylglycerol/phosphotidylinositol ratio [2]. Gunther *et al.* [3] studied the biophysical properties of the surfactant obtained from normal control subjects and IPF patients, and showed that the adsorption and surface-tension-reducing properties were largely lost in virtually all patients with IPF.

McCormack and colleagues [4] hypothesized that the alteration in the surfactant lipid composition changes its biophysical activity, diminishes lung compliance and promotes lung fibrosis. Since SP-A plays an important role in the surface-tension-lowering abilities of surfactant, they measured the SP-A levels in BAL fluid. In addition to reduction in phospholipid content, SP-A levels were also significantly reduced in patients with IPF. The SP-A/phospholipid ratio correlated with disease course over a six-month period and with mortality. In a follow-up study, surfactant levels in BAL fluid were correlated with survival. The mean SP-A/phospholipid ratio was lower in patients with IPF than in healthy volunteers, and the magnitude of reduction was predictive of survival in patients at two years [4]. Others have found a similar reduction in the SP-A levels in patients with IPF but no change in SP-B or SP-D levels [1,3]. Levels of SP-C in BAL fluid of IPF patients are not known. It is clear that there are alterations to the biochemical composition of surfactant in IPF. It is possible that these alterations play an important role in the progression of the disease. Whether the immunological properties of the SPs play a role in the development of the disease needs to be studied.

Other interstitial lung diseases

In sarcoidosis, no substantial changes in surfactant phospholipid profile have been reported in several studies [3]. However, conflicting results have been reported regarding SP-A levels. While van de Graaf *et al.* found unchanged levels of SP-A [5], others have found increased [6] or decreased levels [3]. Although it is possible that the abnormalities may reflect different clinical stages of the disease, it is thought, in general, that sarcoidosis is not associated with major pulmonary surfactant abnormalities [1]. In hypersensitivity pneumonitis, moderate changes in phospholipid profile with reduction in phosphatidylglycerol have been noted [3]. While elevated SP-A levels have been reported in acute disease [7], both low and high levels have been reported in other studies [3,6]. Pulmonary alveolar proteinosis is characterized by the abundance of periodic acid Schiff (PAS) material, which fills the alveolar spaces. In the adult form of the disease, the material is composed of glycoprotein and lipids. SPs A, B, C, and D are all increased in BAL fluid. While the phospholipids have been found to be normal, structural alterations in SP-A and SP-B have been described [8,9].

Serum studies

Recently, Takahashi and colleagues reported the serum levels of SP-A and SP-D, and disease extent in IPF and lung fibrosis associated with scleroderma [10]. In IPF, both SP-A and SP-D concentrations correlated significantly with the extent of alveolitis but not progression of fibrosis. As opposed to lower BAL fluid SP-A levels predicting poor prognosis in other studies, they found that the serum levels were higher in patients who died within three years when compared with patients who lived longer. In scleroderma, the serum levels of SP-A and SP-D were higher in patients with ILD (based on computerized tomography) when compared with patients without any interstitial disease [10,11].

In summary, both lipid and protein components of surfactant can be abnormal in most ILDs, particularly IPF, and there is evidence to suggest that SP abnormalities may be related to survival in specific diseases. Are these changes genetic or do they merely reflect prior tissue damage? An understanding of the genetics of the underlying lung disease in general, and SP expression in particular, may be important in defining susceptibility to and progression of these conditions.

Genetics of interstitial lung disease

The development of ILDs is thought to occur in genetically susceptible individuals, following exposure to a variety of potential environmental triggers. Support for a genetic influence in the development of ILDs comes from two types of observation. First, there is variable susceptibility to environmental causes, and second, familial disease has been reported in most ILDs, including sarcoidosis, IPF, alveolar proteinosis, Langerhans cell histiocytosis, hypersensitivity pneumonitis and desquamative interstitial pneumonia (see Supplementary Table 1).

Complex diseas s

ILDs are relatively rare and susceptibility does not follow single-gene Mendelian patterns. They are referred to

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genetically as 'complex diseases'. Variations at multiple loci, each exerting variable and relatively small effects, are likely to be involved. Further complications in assigning susceptibility involve the assessment of interactions with environmental factors that are thought to induce a specific clinical phenotype, and the knowledge that interactions between genes and the environment can affect the relationships of severity and progression as well as predisposition to disease. Finally, some conditions, such as IPF, manifest in the later stages of life, making family-association studies difficult. For these reasons, most studies of the genetics of ILDs have applied the direct case-controlled, association-based approach. In such studies, the prevalence of alleles in single-nucleotide polymorphisms (SNPs) in biologically important candidate genes is examined in populations of unrelated affected individuals, and compared with prevalence in unrelated normal controls.

Genetic studies in interstitial lung disease

Very few studies have examined the genetic components predisposing to ILDs and, of these, most have focused on the region of chromosome 6, which incorporates the major histocompatibility complex (MHC) and its associated genes. The consensus from the majority of the studies is that susceptibility to sarcoidosis is associated with HLA-DRB1*03, *11, *12, *13, *14. Other associations include alleles in the genes encoding TAP2, the CC chemokine receptor 2, the angiotensin-converting enzyme and vitamin D receptor [12]. Polymorphisms in the fibronectin gene [13] and the HLA-DPB1*1301 allele [14] have been associated with fibrosing alveolitis in the context of systemic sclerosis. Susceptibility to IPF has not been associated with polymorphisms in TNFα, LTα, TNF receptor II and IL-6 genes [15], nor with polymorphisms in the IL-8 and IL-8 receptor (CXCR-1 and CXCR-2) genes [16]. A reported association with the IL-1 receptor-antagonist gene [17] was not confirmed in a subsequent study.

Polymorphisms in the surfactant genes

The genes for the hydrophilic proteins SP-A1, SP-A2 and SP-D have been mapped to human chromosome 10q22-q23.1. The SP-A1 gene is telomeric to the SP-A2 and SP-D genes: the SP-A2 and SP-D genes are located 36 kb and 130 kb respectively from SP-A1. Both SP-A1 and SP-A2 genes have a number of 5′-untranslated region exons that splice under genetic control in different configurations to produce a number of alternatively spliced functional variants [18]. Furthermore, there are a number of polymorphisms within the coding region of the genes that result in amino acid substitutions [19]. In the SP-A1 gene there are five exonic polymorphisms, which correspond to amino acid (aa) positions 19, 50, 62, 133 and 219 of the protein. Two of these are silent (62 and 133), while the others result in a non-conservative amino acid substitution (Ala19→Val, Leu50→Val and Arg219→Trp). In the SP-A2 gene, there are four exonic polymorphisms (Thr9→Asn, Pro91→Ala and Lys223→Gln); the polymorphism at position 140 is silent. Nineteen haplotypes have been identified in the SP-A1 gene (designated 6A to $6A^{20}$), and 15 haplotypes have been identified in the SP-A2 gene (designated 1A to $1A^{13}$) [19]. Of these haplotypes, the most frequent are the SP-A1 ($6A^2$) and SP-A2 ($1A^0$) haplotypes. These two haplotypes comprise the following amino acids: SP-A1 ($6A^2$: Val19/Val50/Arg219) and SP-A2 ($1A^0$: Asn9/Ala91/Gln223). In functional studies, these haplotypes correlated with low or moderate mRNA levels [18]. In the SP-D gene there are two exonic polymorphisms that result in substitutions: Thr11→Met and Thr160→Ala [19].

Genes mapped to human chromosomes 2p12-p11.2 and 8p21 encode the hydrophobic proteins SP-B and SP-C respectively. Several SNPs have been identified in the SP-B gene. Four of these polymorphisms, which reside in the 5′ flanking region, intron 2, exon 4 and 3′ untranslated regions of the gene, have the potential to affect function [20]. The exonic polymorphism substitutes residue 131 (Thr→Ile). There is also a variable nucleotide tandem repeat region , which is highly polymorphic, within intron 4 of the SP-B gene [21]. For the SP-C gene, there may be several SNPs as there are a number of variations between published SP-C sequences [22]. Figure 1 shows the intron/exon structure of the SP genes with the locations of polymorphisms discussed above.

Mutations in the surfactant genes

A locus is considered polymorphic if the less frequent allele has a population frequency of at least 1% and heterozygosity frequency of at least 2%. Below these frequencies, nucleotide variations are allelic variants or, if very rare, they are described as mutations [23]. A number of mutations have been identified in association with hereditary surfactant deficiencies. The predominant, but not exclusive, mutation responsible for SP-B deficiency involves a substitution of a GAA nucleotide triplet for a single C in codon 121, which causes a frameshift and a premature termination signal and also interferes with SP-C processing [24]. Mutations in the SP-B gene are also responsible for SP-B deficiency in congenital alveolar proteinosis [25]. Until the recent work published by Nogee *et al.* [26], there were no data on SP-C mutations and lung disease.

SP-C gene variations in ILD

Nogee *et al.* [26] recently reported an association between a mutation in the SP-C gene and ILD. A full-term baby was born to a woman with a history of desquamative interstitial pneumonia, which was diagnosed when she was one year old and had been treated with corticosteroids up to the age of 15. The baby was normal at birth but developed respiratory symptoms at six weeks of age. Lung biopsy revealed cellular, or non-specific, interstitial pneumonia. The infant improved with oxygen and cortico-

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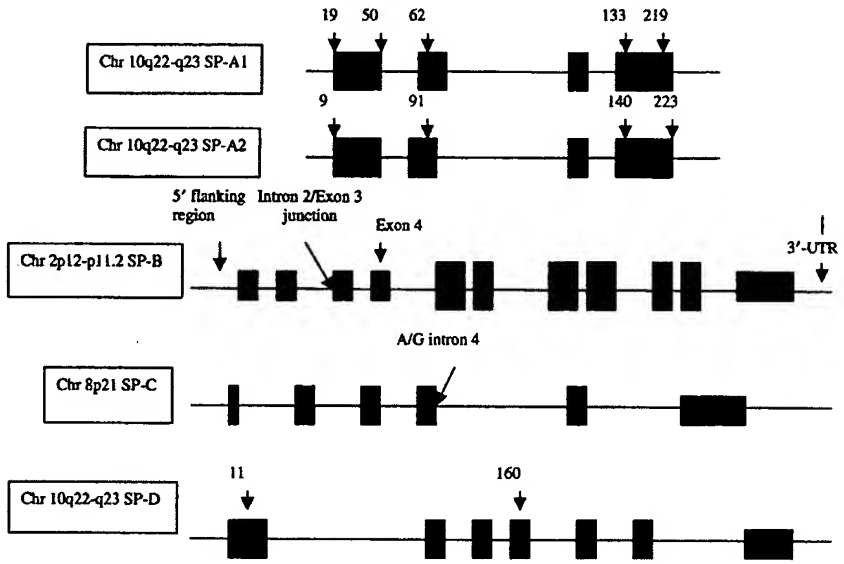
Figure 1



The figure shows the location of the surfactant gene polymorphisms. Exons are represented by black boxes and introns by straight lines (drawn to scale). The numbers refer to the positions of the amino acids in the proteins, as discussed in the text. Chr, chromosome; UTR, untranslated repeat.

steroid therapy. The mother's lung disease worsened and she died of respiratory failure.

Genetic analysis showed a mutation in one allele of the SP-C gene. The heterozygous substitution of A to G was located in the first base of intron 4, abolishing the normal donor splice site and resulting in the skipping of exon 4 and the deletion of 37 amino acid residues in the SP-C precursor protein. Abnormal protein structure is known to result in abnormal tertiary structure and transport. Mature SP-C was completely absent from the BAL fluid and lung tissue of the patient and might have resulted from this aberrant folding and transport. The complete absence of protein with the mutation of a single allele is possibly due to a dominant–negative effect, in which the mutant allele suppresses production of the normal allele [26].

The authors subsequently studied the SP-C gene in 34 infants with nonfamilial chronic lung disease of unknown origin (Nogee *et al.*, personal communication, 2001). They were able to identify mutations of the SP-C gene in 11 infants, which resulted in a phenotype similar to that of the index patient. The occurrence of a *de novo* mutation that is functionally identical to a familial mutation strongly supports the hypothesis that the mutations were causally related to the lung disease. This suggests that SP-C is necessary for normal lung function in the postnatal period.

Surfactant SNP disease-association studies

No other studies have examined surfactant-gene polymorphisms in the context of ILDs, although these have been evaluated in other pulmonary conditions. In a recent publication [27], the SP-B Thr131→Ile polymorphism was found to be associated with the acute respiratory distress syndrome. Alleles in the SP-B variable nucleotide tandem repeat region and SP-A polymorphisms have been reported to be associated with the infant respiratory distress syndrome [21,28], but more recent data suggest that genetic susceptibility to infant respiratory distress syndrome is dependent on SP-A alleles in the context of SP-B Thr131 homozygosity [29]. The SP-A1 polymorphism $6A^6$ is also over-represented in infants with bronchopulmonary dysplasia [30].

Conclusion

In summary, genetic variations are factors in determining the development and severity of many ILDs. Surfactant plays an important role in lung physiology and defense, and surfactant gene variations have been associated with several lung diseases. The recent finding of surfactant gene variations in familial and nonfamilial ILD opens up a new area for more detailed analysis, to explore whether these variations play a role in a wider range of ILDs.

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Supplementary material

Supplementary Table 1

Studies of genetic polymorphisms in various interstitial lung diseases

Disease	Associated gene products	References
Idiopathic pulmonary fibrosis (IPF)	HLA	[S1]
	IL-1, TNF-alpha	[15,17,S2]
	Chemokines	[16]
	ACE	[S3]
Sarcoidosis	HLA	[S4–S21]
	ACE	[S22–S29]
	TNF-alpha	[S30–S34]
	Chemokines	[S35,S36]
	NRAMP	[S37]
	Mannose-binding lectin	[S38]
	Vitamin-D receptor	[S39,S40]
	IL-1	[S41]
Fibrosing alveolitis in scleroderma	Fibronectin	[13]
	HLA	[14]
Extrinsic allergic alveolitis	TNF-alpha	[S42]
Langerhan's cell histiocytosis	TNF-alpha	[S43]

ACE, angiotensin-converting enzyme; IL-1, interleukin-1; NRAMP, natural resistance-associated macrophage protein; TNF, tumor necrosis factor.

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Attachment 5

Progressive lung disease and surfactant dysfunction with a deletion of surfactant protein C gene

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Running title: Disrupted surfactant function with SP-C mutation

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Abstract

Mutations in the surfactant protein-C (SP-C) gene are responsible for familial and sporadic interstitial lung disease (ILD). The consequences of such mutations on pulmonary surfactant composition and function are poorly understood. To determine the effects of a mutation in the SP-C gene on surfactant, we obtained lung tissue at the time of transplantation from a 14 month old infant with progressive ILD. An in-frame 9-base pair deletion spanning codons 91-93 in Exon 3 of the SP-C gene was present on one allele; neither parent carried this deletion. SP-C mRNA was present in normal size and amount. By immunofluorescence, proSP-C was aggregated within alveolar Type II cells in a compartment separate from SP-B. In airway surfactant, there was little or no mature SP-B or SP-C; SP-A content was increased. Minimum surface tension was increased (20mN/m, normal <5 mN/m). Type II cells contained normal and disorganized appearing lamellar bodies by electron microscopy (EM). This spontaneous deletion on one allele of the SP-C gene was associated with sporadic ILD and abnormalities in surfactant composition and function. We propose that a dominant negative effect on surfactant protein metabolism and function results from aggregation of misfolded proSP-C and subsequent cell injury and inflammation.

Key words: pulmonary surfactant, protein misfolding, surfactant proteins, interstitial lung disease, infant

Abbreviations

BAL – bronchoalveolar lavage

BPD – bronchopulmonary dysplasia

EM– electron microscopy

ER – endoplasmic reticulum

ILD – interstitial lung disease

PAP – pulmonary alveolar proteinosis

PC – phosphatidylcholine

PCR – polymerase chain reaction

PE – phosphatidylethanolamine

PG – phosphatidylglycerol

PI – phosphatidylinositol

SP-A, SP-B, SP-C, SP-D – surfactant proteins A, B, C, D

Introduction:

Pulmonary surfactant is a phospholipid–protein complex that is synthesized by alveolar Type II cells and maintains alveolar expansion at end expiration. Of the four surfactant associated proteins (SP-A, SP-B, SP-C, and SP-D), surfactant proteins B and C interact with the phospholipid components in a tightly coordinated itinerary of synthesis, secretion, film formation, and recycling (1).

SP-C is a hydrophobic peptide encoded by an approximately 3kb, 6-exon gene on the short arm of human chromosome 8. The synthesis and post-translational processing of SP-C have been recently reviewed (2). Alternative splicing results in a 191 or 197 amino acid, 21 kD propeptide that is proteolytically cleaved to a 35 amino acid ~4 kD peptide. The mature peptide is encoded within Exon 2 of the SP-C gene and is palmitoylated at cysteine residues at positions 5 and 6. Complete biosynthesis requires sequential endoproteolytic cleavages of the SP-C propeptide and depends upon oligomeric sorting and targeting to subcellular processing compartments distal to the Golgi. The mature SP-C domain contained within the propeptide (residues 24-58) functions as a signal-anchor sequence effecting endoplasmic reticulum (ER) translocation, establishing a type II ($NH2_{cytoplasm}/COOH_{lumen}$) integral membrane orientation, and facilitating homomeric association during sorting (3). The NH_2 flanking propeptide contains a functional targeting motif necessary for directing proSP-C from the endoplasmic reticulum (ER) to distal processing compartments, the multivesicular and lamellar bodies (4,5). Deletion or alteration of this region results in ER retention. Conversely, while the COOH flanking propeptide is not required for targeting or post-translational processing, removal or alteration of one or more cysteine residues from the COOH flanking propeptide results in

mutant protein accumulation in a novel juxtanuclear compartment, the aggresome (6). SP-B is integrally involved in this metabolic itinerary in that infants and mice genetically deficient in SP-B exhibit a 6-12 kD incompletely processed proSP-C peptide in both intracellular and extracellular surfactant (7, 8).

Mice genetically deficient in SP-C exhibit strain dependent pulmonary pathology. Swiss black SP-C null mice have no pulmonary pathology but have surfactant that is unstable at low volumes (9). In contrast, 129/Sv SP-C null mice develop significant lung remodeling and increases in phospholipid pools but no alteration in the expression of SP-B (10). Recently, mutations in the SP-C gene corresponding to the COOH terminus of proSP-C have been associated with familial and sporadic interstitial lung disease in adults and children (11-13). In contrast to the recessive null mutations seen in SP-B deficient infants, all affected patients to date possess a mutation on only one allele of the SP-C gene, suggesting a dominant negative effect of the mutant SP-C, which has been recently demonstrated in vitro (14). The histologic and clinical expressions of these mutations have been variable with children exhibiting chronic pneumonitis of infancy or non-specific interstitial lung disease (ILD) and adults exhibiting usual interstitial pneumonitis, desquamative interstitial pneumonitis or idiopathic pulmonary fibrosis. The age of onset has ranged from 6 weeks to over 50 years. The mechanisms of cell injury and lung disease are thought to result from accumulation of incompletely processed/misfolded SP-C protein that is toxic to cells *in vitro* (12,14) .

Lung transplantation has been successful for infants with inherited SP-B deficiency and has provided the opportunity to elucidate pulmonary surfactant expression, composition and function (15, 16). This opportunity has not previously been

available for subjects with mutations in the SP-C gene. Therefore, to address the hypothesis that pulmonary surfactant expression, composition, and function are disrupted in patients with mutations in the SP-C gene, we analyzed lung tissue and BAL fluid obtained from an infant with a novel mutation who underwent lung transplantation for progressive ILD.

Case:

This 4.1 kg female was the full term product of an uncomplicated pregnancy. She is the first child of these two parents. She was healthy until 3 months of age when she presented with growth failure, difficulty feeding, and diffuse interstitial infiltrates on chest radiograph. Open lung biopsy at 6 months of age revealed interstitial pneumonitis without a defined etiology (Fig 1). Genetic analysis at 9 months of age revealed a 9 basepair deletion in Exon 3 of the SP-C gene. Progressive decline in pulmonary function prompted bilateral lung transplantation at 14 months of age. At 30 months of age, she was breathing ambient air, gaining weight and had a developmental stage of approximately 24 months. Both parents were healthy and there was no family history of pulmonary disease, specifically interstitial lung disease or idiopathic pulmonary fibrosis.

Methods:

Genetic analysis:

Genomic DNA was prepared from blood leukocytes using a commercially available kit (Puregene, Gentra Systems, Minneapolis, MN). Amplimers spanning exons 1 to 2 (genomic positions -143 to 996) and exons 3 to 6 (genomic positions 1212 - 2522) of the SP-C gene were generated by PCR from genomic DNA of the child and parents and analyzed by direct sequencing of the PCR products. The SP-B gene was amplified using primers and conditions as previously described (17). PCR conditions were the same for both the SP-B and SP-C genes, with an annealing temperature of 60°C, and cycle sequencing was performed as previously described. Patient SP-C gene sequence was compared to previously published SP-C sequence. DNA from 50 adults without known lung disease were analyzed anonymously for the mutation found in the patient by direct analysis of PCR products on 3% metaphor agarose gels.

RNA preparation, Northern blot hybridization, and in situ hybridization.

Total RNA was extracted from frozen lung tissue obtained at transplantation by the acidic guanidinium thiocyanate method (18). OD_{260}/OD_{280} ratios were 1.80 to 1.94 for all samples. Contents of specific mRNAs were analyzed by Northern blot hybridization and scanning densitometry using [^{32}P]-labeled human cDNA probes for SP-A (900 bp), SP-C (875 bp), and ß-actin (1.9 Kb), prepared by random primer labeling with [^{32}P]dCTP and the Ready-To-Go kit (Pharmacia), as described (19). For Northern analyses, total RNA (10 µg applied per lane) was run on 1.2% agarose-formaldehyde gels

and RNA species were transferred to nitrocellulose membranes (Duralose-UV, Stratagene) which were baked, and hybridized with ^{32}P-labeled cDNA probes. Autoradiography films were scanned with a densitometer and data expressed as density units per μg RNA applied.

In situ hybridization was performed on paraffin embedded lung tissue as described previously (20). Briefly, antisense and sense [^{35}S]cRNA probes to SP-A, SP-B, and SP-C were synthesized as described using ^{35}S-labeled UTP and ^{35}S-labeled CTP. After deparaffinization, sections were hybridized with antisense and sense ^{35}S-labeled cRNA probes at a specific activity of 4 X 10^7 counts-min^{-1}-ml^{-1} of hybridization solution. After exposure for 7 to 10 days at 4°C, slides were developed, counterstained with hematoxylin and eosin, and examined under bright-field and dark-field illumination.

Surfactant composition and function

At the time of lung transplantation, sections of the explanted lung were frozen immediately upon removal and bronchoalveolar lavage (BAL) was performed upon one lobe of the explanted lung. Tissue, BAL, and tracheal aspirate fluid used as controls came from transplant donor lungs, premature infants requiring intubation beyond 7 days of life, and infants who required lung transplantation for refractory lung disease of undetermined etiology. SDS polyacrylamide gel electrophoresis and protein blotting were performed on homogenates of lung tissue and on the BAL fluid as previously described (7,16). Surface tension measurements and immunodot assays for SP-A, SP-B, and SP-C were performed on serial dilutions of large aggregate surfactant-rich fraction isolated by centrifugation of the BAL (27,000 x g for 60 minutes) (21). Surface properties were

assessed at 37^0C in humidified air with a pulsating bubble surfactometer (Electronetics Corp, Buffalo, NY) after adjusting the phospholipid concentration to 1.5 mg/ml.

After chloroform methanol extraction of BAL fluid with addition of 14:0/14:0 phosphatidylcholine (PC, 25 μmol), 14:0/14:0 phosphatidylglycerol (PG, 3 μmol) and phosphatidylethanolamine (PE, 1.5 μmol) as the internal standards, electrospray ionization (ESI) mass spectrometric analyses were performed to assess phospholipid composition using a Finnigan (San Jose, CA) TSQ-7000 triple stage quadrupole mass spectrometer. PC was quantified as sodiated ion species in the positive-ion mode using constant neutral loss scanning of 183 (22). To quantify PG, PE, and phosphatidylinositol (PI) species in the negative-ion mode by ESI/MS, 0.1% ammonium hydroxide in methanol was added to the lipid mixture, which was re-extracted with chloroform, dried under nitrogen, re-dissolved in chloroform/methanol (1/3, vol/vol) and subjected to ESI analysis. The structures of the molecular species of the phospholipids were identified by the product-ion spectra of the $[M + Na]^+$ ions for PC, and of the $[M - H]^-$ ions for PG, PE and PI as previously described (23,24).

Antibodies

Production and characterization of polyclonal antisera directed against SP-A, SP-B, proSP-B, and SP-D have been described (25-28). Monospecific polyclonal rat and rabbit proSP-C antisera produced against synthetic peptide proSP-C peptides have been previously characterized (29,30). Anti-NPROSP-C (Met^{10} to Glu^{23}) recognizes proSP-C_{21} and all major intermediates but does not recognize mature SP-C. Antibody to mature SP-C was provided by ALTANA Pharma AG (Konstanz, Germany) and was generated

against recombinant human SP-C containing Phe substituted for Cys residues 3 and 4 and Ile for Met at residue 32. On protein blotting, this antibody recognizes mature SP-C of approximately 4 kDa and has very weak immunoreactivity for precursor forms of SP-C (31).

Pulmonary histology, immunohistology and ultrastructure

Immunohistochemical analyses using polyclonal antisera directed against SP-A, SP-B, proSP-C, and SP-D were performed on formalin-fixed, paraffin-embedded tissue as previously described (11,32). Fluorescence immunocytochemistry of fixed lung sections was done using anti-NPROSP-C as previously described (29). Lung tissue obtained at transplantation was fixed in 3% glutaraldehyde and prepared for electron microscopy as previously described (33).

Informed consent was obtained from the family and the study was approved by the institutional review boards of the respective institutions.

Results:

Genetic analysis:

Sequence analysis demonstrated a 9-basepair deletion (sequence ACT GCC ACC) on one allele in Exon 3 of the SP-C gene corresponding to codons 91-93 in the proSP-C peptide. This in-frame deletion would result in the deletion of three amino acids (Thr Ala Thr) in the proximal COOH flanking domain of the SP-C proprotein. This mutation was not present in either parent, suggesting that this was a de novo mutation (Figure 2). The deletion was also not observed in 100 control chromosomes, indicating that it is not a common polymorphism.

No variations from the known SP-B gene sequences or single nucleotide polymorphisms were identified.

Transcript expression

Northern blot analysis of RNA from the explanted lung tissue showed a single SP-C band of the expected size with a staining intensity similar to comparison samples from infants with lung disease (inherited SP-B deficiency and bronchopulmonary dysplasia, not shown). This technique could not resolve transcripts differing by 9 basepairs, however a band corresponding to each allele was observed with RT-PCR using primers spanning the site of the deletion (not shown). SP-A transcript in the patient was the expected size with somewhat stronger staining intensity compared to other samples tested. In situ hybridization revealed the expected amount and distribution of SP-A, SP-B, and SP-C mRNA expression (data not shown).

Surfactant protein expression

By Western blot analysis, mature SP-C was not detected in large aggregate surfactant prepared from the patient's BAL but was present in control samples (Figure 3). A strong band for SP-A was observed in the patient's surfactant and was increased relative to premature infant controls by immunodot analysis (Table) and immunohistochemistry (Figures 1 and 4). By Western blot analysis, no immunoreactive SP-B bands were seen in BAL. However, mature SP-B was present in the patient's lung homogenate (data not shown), was low in large aggregate surfactant by immunodot analysis, and was weakly detected in Type II cells by immunohistochemistry (Figure 4). ProSP-B staining was found both within Type II cells and in alveolar spaces of the patient, however was not significantly different from that of lung injury controls (data not shown). Staining with anti NPROSP-C showed a stronger and more localized signal in the perinuclear region of the patient's Type II cells than in normal and lung disease controls (Figure 5). In the controls, SP-B and proSP-C had similar distribution in cytosolic vesicles suggesting co-localization in multivesicular or lamellar bodies. In contrast, in the patient, proSP-C and SP-B had distinctly different distributions suggesting localization in different compartments in the alveolar type II cell. These findings were supported by routine immunostaining of formalin-fixed, paraffin-embedded lung tissue for mature SP-B and proSP-C (data not shown).

Function and phospholipid composition of large aggregate surfactant

The surfactant fraction of the patient was compared to that of six intubated premature infants (samples selected for normal surface tension properties) and adults

with pulmonary alveolar proteinosis (PAP). As assessed in a pulsating bubble surfactometer, the minimum surface tension of the patient's sample was elevated, indicating in vitro surfactant dysfunction (Table). The content of the total protein relative to phospholipid was increased in the patient's surfactant compared to infant controls. PC comprised 78% of the phospholipid (controls 84 $\pm$ 1%), 46% of which was dipalmitoyl PC, which was not significantly different from controls (43 $\pm$ 2% of total PC). The relative proportion of PI was increased (8% vs 2 $\pm$ 1%, patient and controls, respectively), the significance of which remains to be determined.

Histology and ultrastructure

The histologic appearance of the explanted lungs resembled that of the biopsy obtained 8 months earlier, with increased interstitial fibrosis and focal acute pneumonia. There were also focal areas with weakly periodic acid-Schiff positive granular material in the airspaces suggestive of alveolar proteinosis (not shown). Electron microscopy of the explanted lung revealed type II pneumocytes with abundant lamellar bodies in apical cytoplasm, some of which were disorganized (Figure 6). In addition, a minority of type II cells contained large disorganized perinuclear membranous structures. A rare cell contained cytoplasmic dense membrane bound and multivesicular structures, however, pneumocytes with numerous multivesicular bodies characteristic of SP-B deficiency were not seen (34).

Discussion

In this report of an infant with progressive and severe ILD associated with a spontaneous 9-basepair deletion on one allele of the SP-C gene, proSP-C accumulated in an intracellular compartment separate from SP-B and mature SP-C was not detected in large aggregate surfactant. These findings suggest that the trafficking and/or processing of normal proSP-C was disrupted by the presence of mutated proSP-C, altering surfactant composition and function.

Inherited mutations in the SP-C gene have been identified in individuals with familial ILD and de novo mutations have been identified in individuals with sporadic ILD, suggesting that the mutations are causally linked with disease (11-13). This infant's novel deletion in the presence of ILD and the absence of the mutation and disease in the parents further strengthens this association. In addition, similar to all previously reported cases, the mutation was present on only one allele, suggesting a dominant negative effect.

The exact mechanisms of lung disease resulting from mutations in the SP-C gene remain to be fully elucidated. However, the information derived from our current studies supports previous *in vitro* studies and provides insights into other mechanisms that may promote respiratory dysfunction in these patients. First, we demonstrated aggregation of proSP-C within Type II cells in explanted lung tissue, similar to findings with transfected lung epithelial cells. A549 cells transfected with constructs containing mutated proSP-C sequences have demonstrated that alterations in the COOH terminus of rat SP-C (deletion of Leu 185-Ile194) or human SP-C (deletion of Exon 4: Leu 110-Gln 144) result in accumulation of the mutant proSP-C in aggresomes (6,14). In addition, transfection of SP-C cDNA containing the point mutation seen in a family with ILD (Exon 5 + 128

T>A) into mouse lung epithelial cells resulted in cytotoxicity (12). In the subject of this report, the absence of mature SP-C in the large aggregate surfactant and the juxtanuclear accumulation of proSP-C are consistent with a dominant negative effect of a presumably misfolded peptide that prevents normal proSP-C trafficking into the multivesicular and lamellar bodies (6). It has been speculated that the aggregation of misfolded proSP-C may interfere with the "unfolded protein response" and result in toxic gain of function similar to that seen in mice that over-express SP-C, or in patients with Alzheimer disease, α1-antitrypsin deficiency and cystic fibrosis (35,36). In addition, amino acids 94-191 of the COOH terminus of SP-C share sequence similarity with propeptide domains of BRI2, chondromodulin-I and CA11, the "BRICHOS" family (37). Mutations in these domains result in dementia, chondrosarcoma, and stomach cancer, respectively, likely due to disrupted peptide processing, targeting, and/or aggregation, mechanisms which appear to be operative with SP-C mutations, as well.

Surfactant dysfunction, not previously reported in patients with SP-C mutations, is another factor that probably contributed to this infant's decline in respiratory function. Whether the decreased surfactant function was specific to disrupted SP-C metabolism or was a more generalized response to cell injury and inflammation is unknown. The lack of mature SP-B and SP-C in the large aggregate surfactant is a prominent finding that would explain lack of surface activity and respiratory dysfunction. Murine lineages conditionally expressing SP-B develop respiratory failure, altered surfactant composition and decreased surfactant function when SP-B levels reached 25% of normal values (38). *In vitro,* surfactant phospholipids in the absence of surfactant proteins have a slow adsorption rate to the surface film and do not achieve low surface tensions; normal

surfactant properties are restored in a dose-dependent fashion by reconstitution of lipids with SP-B (39). Thus, the absence of detectable mature SP-B and SP-C in our patient's surfactant would disrupt the surfactant phospholipid layer and decrease surfactant function. Another possibility is that the misfolded proSP-C itself may inhibit surfactant function, however there is no evidence that this proSP-C is secreted into the alveolar space. The presence of proSP-B immunostaining in the alveolar space, the presence of disorganized lamellar bodies on electron microscopy and apparent intracellular aggregation of proSP-C suggest that the metabolism and assembly of the surfactant complex was disrupted, similar to that seen with SP-B deficient infants (7,16,34,40).

While these alterations in surfactant composition and function may be specific for mutations in the SP-C gene, another possibility is that the alterations are a non-specific response to cell injury. Decreased or absent SP-B and SP-C are features of other lung diseases associated with inflammation such as interstitial lung disease without mutations in the SP-C gene, in adults with acute respiratory distress syndrome, in premature infants with chronic lung disease, and in animals with bleomycin-induced lung injury (32,40,41). Since this infant presumably had normal pulmonary function for several months after birth, we speculate that, like the Swiss black SP-C null mice, surfactant function was normal under normal conditions, but was easily disrupted once challenged with an inflammatory response either from accumulation of proSP-C or from a secondary insult such as aspiration or infection.

This is the first infant known to have a mutation in the SP-C gene to be considered for lung transplantation. In contrast to the known and rapidly lethal outcome for infants with SP-B deficiency for whom discussions with the family about lung

transplantation can be initiated at the time of diagnosis, infants and adults with interstitial lung disease due to mutations in the SP-C gene have more variable, and hence, unpredictable short and long-term outcomes (11,12,15). Thus the consideration for lung transplantation is not as straightforward as that for infants with inherited SP-B deficiency. In this case, the criteria regarding commitment to lung transplantation that are currently applied for other forms of progressive lung disease were applied. Only when it became apparent that the patient's condition was deteriorating and refractory to medical therapy was she listed for transplantation.

In summary, lung transplantation for an infant with ILD due to a mutation in the SP-C gene afforded the opportunity to investigate surfactant composition and function. These studies provide further indication that altered SP-C processing leads to a chronic inflammatory state that disrupts surfactant metabolism and function. Additional experience with infants with mutations in the SP-C gene will permit genotype-phenotype correlations that will help predict the natural history and provide more informed decision-making about the need for lung transplantation.

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Table: Function and surfactant profile of large aggregate surfactant pellet

	Patient BAL	Infant tracheal aspirate	Adult PAP BAL
Minimum surface tension (mN/m)	20.0	3.0 (0-6.1)	12.0 (7.8-15.0)
Total protein (μg/μgPL)	2.5	0.7 (0.2-1.8)	6.0 (3.1-9.1)
SP-A (μg/100μgPL)	15.0 (10.8-21.0)	4.0 (0.3-10.0)	647.0 (506.9-727.0)
SP-B (μg/100μgPL)	0.6 (0.4-1.1)	1.9 (0.6-4.6)	9.4 (4.0-16.6)

Data are mean and range for 4 replicate determinations of the patient sample, 6 samples of premature infant tracheal aspirate, and 4 samples from adults with PAP. Data for SP-A and SP-B are presented as μg per 100 μg total phospholipid (PL) by weight (by phosphorous assay of large aggregate surfactant pellet).

Figure legends

Figure 1. Photomicrographs of lung biopsy obtained at 6 months of age. Hematoxylin and eosin staining (H&E) shows prominent type II pneumocyte hyperplasia and alveolar septal widening with a mild lymphoplasmacytic infiltrate along with smooth muscle extension and fibroblast proliferation. In this field, foamy macrophages and granular material are focally present in alveolar spaces. SP-A and SP-D expression is robust in Type II cells and alveolar material, as seen by staining with polyclonal rabbit anti-human SP-A antibody and polyclonal rabbit anti-mouse SP-D antibody. Pneumocyte hyperplasia, alveolar macrophage accumulation, and cholesterol clefts are prominent in these fields. Scale bars equal 100 microns.

Figure 2. PCR amplification of SP-C exon 3. In the lane corresponding to the patient's DNA (Pt), there is a band at 206 basepairs, corresponding to the allele with the 9 basepair deletion, along with a band migrating at the expected size of 215 basepairs, corresponding to the normal allele. The lanes corresponding to the mother's (M), the father's (F), and an unaffected control's (Con) DNA contain only the 215 basepair fragment band corresponding to the normal allele. The smaller PCR product corresponding to the mutated allele in the patient's DNA was better resolved after digestion of the PCR products with the restriction enzyme*MspI* (right).

Figure 3. Protein immunoblots for SP-A, SP-B and SP-C. Large aggregate surfactant was prepared from BAL of the patient (Lane 1), and tracheal aspirate samples from three premature infants (Lanes 2-4). The amount of total protein applied was 7 μg for all samples. Mature SP-A is observed at approximately 35 Kda. The gels for SP-B and SP-C were run under reducing conditions. Mature SP-B migrates at approximately 8 kDa with a doublet band for two tracheal aspirate samples. Mature SP-C is seen at approximately 4 kDa. In the patient's sample, SP-A was detected, but mature SP-B and SP-C were not.

Figure 4. Immunohistochemical stains for SP-A, SP-B, and proSP-C in lung tissue of the case patient (top row) and a transplant donor (bottom row). The case patient exhibits decreased staining for SP-B and normal to increased staining for proSP-C in Type II cells. SP-A is normal in the Type II cells but increased in the material in the airspaces. Inset: negative control with non-immune serum (NIS). Original magnification 400x for all images except the NIS (100x).

Figure 5. ProSP-C and SP-B expression in lung of a transplant donor, the case patient, and an infant with BPD. Sections were stained with primary polyclonal anti-NPROSP-C (top row) and anti-human mature SP-B (bottom row) and secondary Texas Red anti-rabbit IgG. Fluorescence microscopic images demonstrate intracellular proSP-C aggregates in alveolar type II cells in the case patient, in contrast to the controls in which, despite increased numbers of type II cells, shows proSP-C to be distributed in cytosolic vesicles. Staining for SP-B demonstrated distribution in cytosolic vesicles in all samples. Scale bars equal 10 microns.

Figure 6. Electron microscopy of the patient's explanted lung tissue shows hyperplastic type II pneumocytes with numerous lamellar bodies in apical cytoplasm, some of which appear normal (arrow) and others which appear to be disorganized (*). Scale bar equals 1 micron.

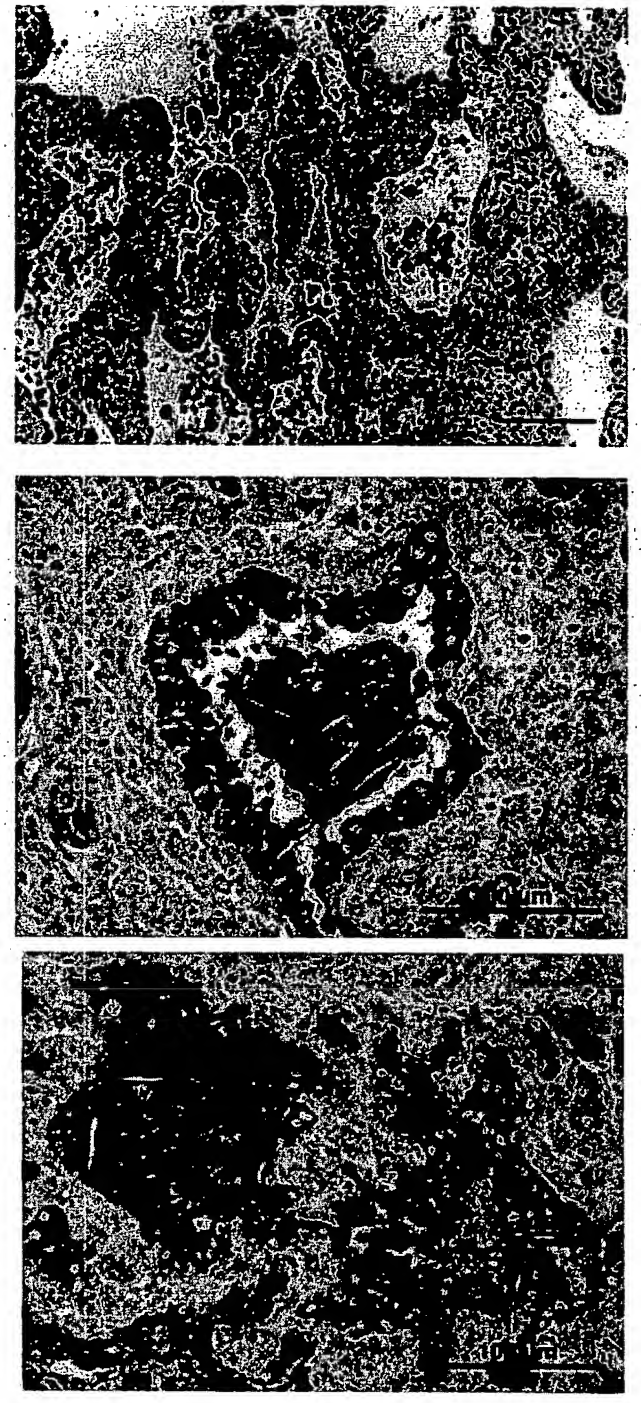
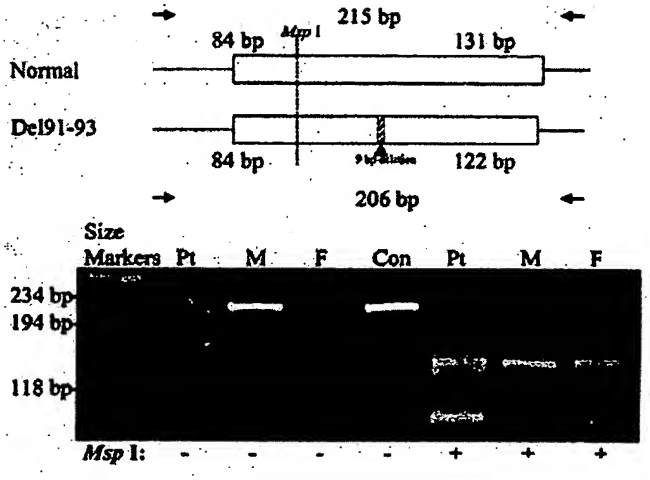


Figure 1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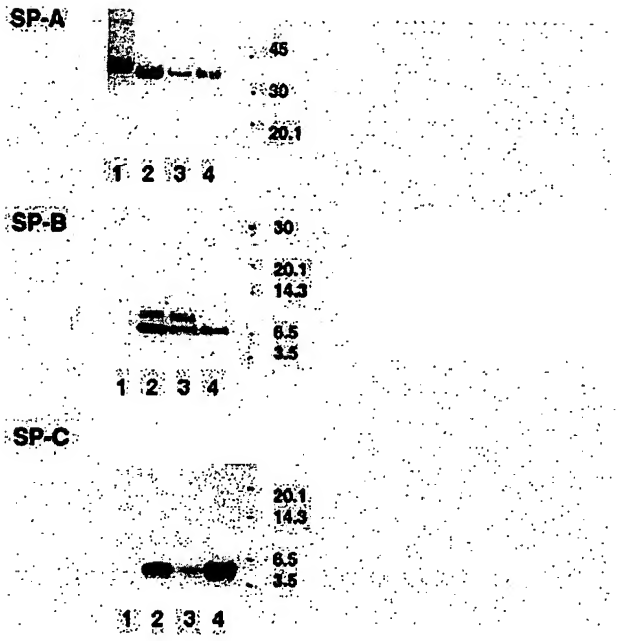
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6.5
3.5
1 2 3 4
SP-C
20.1
14.3
6.5
3.5
1 2 3 4

Figure 3

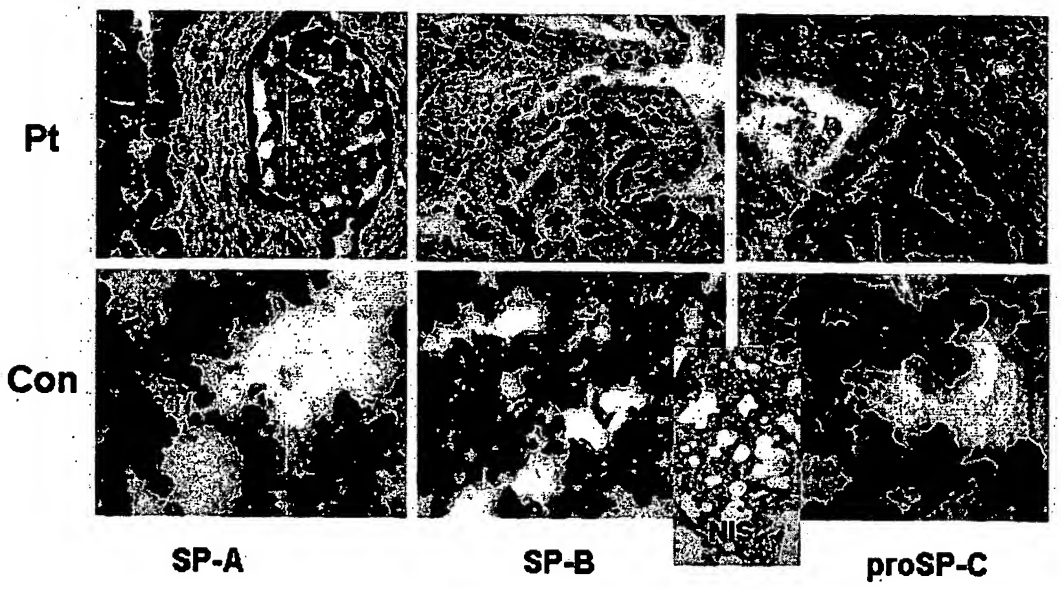

Figure 4

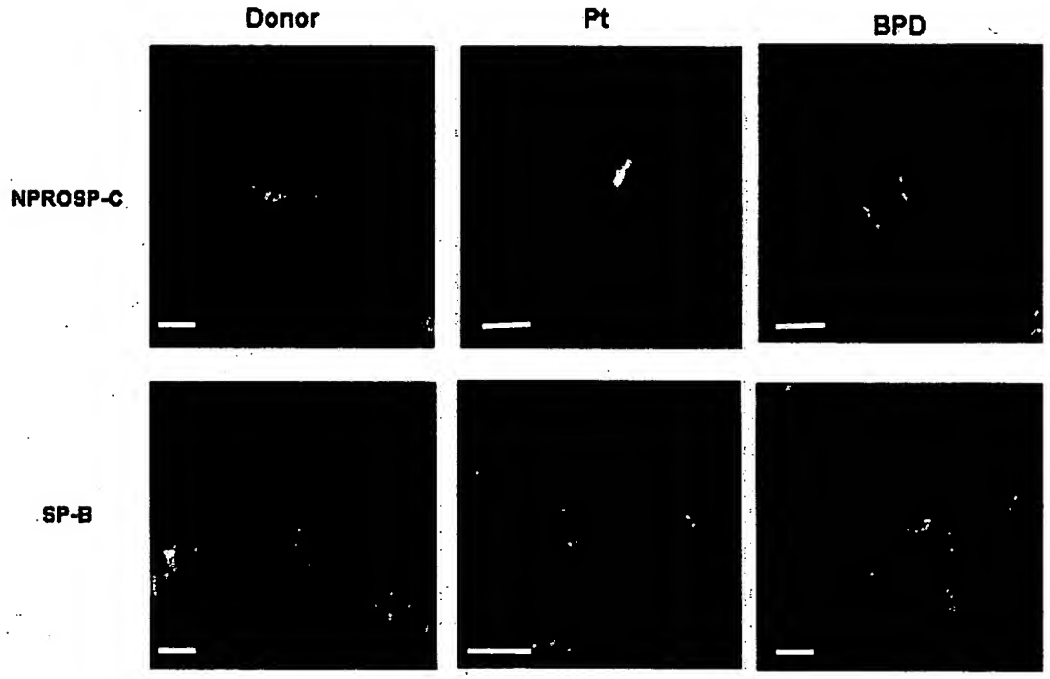

Figure 5

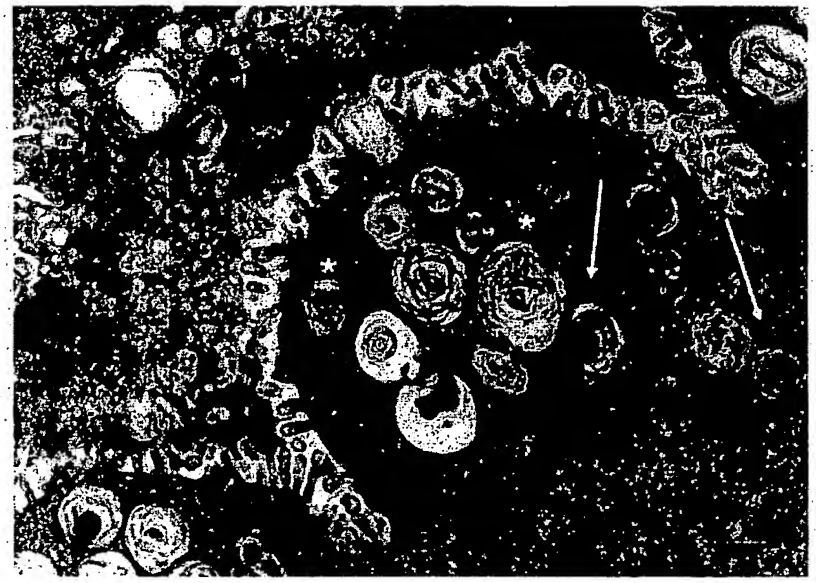

Figure 6